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**ENGINEERING MESENCHYMAL STEM CELL SHEETS ON FUNCTIONAL  
POLYELECTROLYTE MULTILAYER SUBSTRATES**

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## ABSTRACT

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Cell sheet engineering is a novel and effective means of forming tissue substitutes in regenerative medicine. This technology allows for the manufacturing of cell sheets for either direct transplantation or the assembly of three-dimensionally organized tissue-mimicking structures. In the last decade, cell sheet engineering emerged as a promising alternative to injected cell delivery and biomaterial vehicles. Cell sheets manufactured on thermosensitive polymer coatings are already successfully employed in clinical applications such as human corneal reconstruction and treatment of human myocardial dysfunction.

The main goal of my thesis was to develop an alternative, electrochemically responsive surface coating for the site-specific release of cell sheets by applying of micro-electrical currents. In context of my work, polyelectrolyte multilayer (PEM)-based platforms should be used for the synthesis of cell sheets composed of human stem / progenitor and end-differentiated cells.

In the first part of my thesis, I determined the critical parameters for the isolation and long-term cultivation of human term placenta-derived mesenchymal stem cells (PD-MSCs). My results revealed that the isolated cells were of maternal origin and possessed very high proliferation and very low mortality rates up to passage twenty. The phenotypic profile of PD-MSC-isolates fulfilled the recently defined minimal criteria for the determination of mesenchymal stem cells (MSCs) and remained constant throughout continuous sub-confluent culture. The plasticity of PD-MSCs allowed their differentiation into adipogenic, chondrogenic, and osteogenic lineages as well as endothelium.

In the second part of my thesis, I designed and optimized a PEM-based platform for the growth and intact peeling of cell sheets generated from different human cell types. Therefore, I established PEM substrates of a constant thickness with variations in rigidity. As a preliminary experiment, I used the assembly composed from nine layer-pairs of positively-charged poly-L-lysine (PLL) and negatively-charged hyaluronic acid (HA). The use of such polymers allowed for the construction of soft coatings and has been previously described as an attractive system for culturing different cell types. Moreover, the coating stiffness was adjusted by modulating the heterobifunctional

crosslinkers present. For the assembly of stiff coatings, I used a film comprised of positively-charged poly-(allylamine)-hydrochloride (PAH) and negatively-charged poly-(styrene)-sulfonate (PSS). The potential of such coatings in cell sheet formation was previously demonstrated by endothelial cell cultivation. I demonstrated that the stiffness of the PEM substrate plays a critical role in the adhesion, spreading, and growth of human cells. Although the soft substrate supported the adhesion of the control murine muscle cell line C2C12, it only provided a minimal support for the adhesion of human cell types such as placenta-derived MSCs (PD-MSCs), adipose tissue-derived MSCs (AT-MSCs), human muscle cells (HMCs), human umbilical cord endothelial cells (HUVECs), and human late outgrowth endothelial cells (OECs). In contrast, the semi-stiff and stiff substrates were deemed suitable in culturing all of the tested human cell types and allowed for the complete mesodermal differentiation of stem cell sheets generated from AT-MSCs and PD-MSCs.

In the final part of my thesis, I established the optimal conditions for the generation and detachment of PD-MSC sheets. In order to find a PEM architecture that minimally affected the cell sheet growth, I decided to utilize stiff (PAH/PSS) coatings. Such coatings supported the successful formation of PD-MSC sheets without any chemical modification. They also allowed for cell sheet detachment mediated either electrochemically by applying the micro-electrical current about 1.8 mV or by decreasing the environmental pH to 4. The viable PD-MSC sheets that detached from the (PAH/PSS) platform were able to adhere on the fresh TCPS substrates and could be successfully differentiated towards adipogenesis and osteogenesis.

In summary, my thesis explored and implemented a PEM-based platform as substrates for cell sheet engineering and differentiation. Comparable with costly classical thermosensitive platforms, economically priced PEM based platforms allowed formation of cell sheets from different human stem / progenitor and adult cell types during a very short time period. Moreover, charge and pH-mediated peeling of cell sheets supported by PEM platforms are not only faster, but easier to automatize than thermosensitive platforms as well. My results lead me to believe that PEM based platforms indeed have potential as substrates for cell sheet engineering. They can be

used for manufacturing single stem cell sheets geared for direct transplantation or for the generation of tissue-like structures destined for drug screening applications.

## ZUSAMMENFASSUNG

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Die Engineering der Zellschichten oder „*Cell Sheet Engineering*“ hat sich zu einer modernen und effektiven Technik für die regenerative Medizin entwickelt. Diese Technologie ermöglicht die Herstellung von Zellschichten für die direkte Transplantation oder den Zusammenbau von dreidimensional organisierten, gewebeähnlichen Strukturen. Im letzten Jahrzehnt wurde „*Cell Sheet Engineering*“ eine vielversprechende Alternative zur Verabreichung von Zellen durch Injektion oder mittels Biomaterialien. Zellschichten, welche auf wärmeempfindlichen Polymerbeschichtungen hergestellt werden, können bereits erfolgreich in klinischen Anwendungen wie beim Wiederaufbau von Hornhaut oder der Behandlung von menschlicher Myokarddysfunktion eingesetzt worden.

Das Hauptziel meiner Arbeit war die Entwicklung einer alternativen, auf elektrochemische Signale reagierenden Oberflächenbeschichtung, welche die ortsspezifische Freisetzung von Zellschichten durch Anlegen einer Mikro-elektrischer Ladung erlaubt. Im Rahmen meiner Arbeit wurde eine Polyelektrolyt-Multischicht (PEM)-basierte Plattform verwendet, um Zellschichten aus menschlichen Stammzellen und enddifferenzierten Zellen herzustellen.

Im ersten Teil meiner Dissertation habe ich kritische Parameter für die Isolation und langfristige Kultivierung von mesenchymalen Stammzellen aus humaner Plazenta (PD-MSCs) bestimmt. Meine Ergebnisse zeigen, dass die isolierten Zellen mütterlichen Ursprungs sind und in den ersten zwanzig Passagen eine sehr hohe Proliferationsrate und gleichzeitig eine sehr niedrige Sterblichkeitsrate aufweisen. Die isolierten PD-MSC erfüllen mit ihrem phänotypischen Profil die kürzlich definierten Minimalkriterien für die Bestimmung von mesenchymalen Stammzellen (MSCs) und bleiben während kontinuierlicher sub-konfluenter Kultur phänotypisch stabil. Die PD-MSCs weisen eine Plastizität auf, die deren Differenzierung in Richtung Adipogenese, Chondrogenese und Osteogenese sowie Endothelium erlaubt.

Im zweiten Teil meiner Dissertation habe ich eine optimierte PEM-basierte Plattform zur Herstellung von intakten Zellschichten entworfen und dahingehend optimiert, dass aus unterschiedlichen menschlichen Zelltypen Zellschichten erzeugt und von der Oberfläche abgelöst werden können. Dazu habe ich PEM Substrate mit konstanter Dicke

und variabler Steifigkeit hergestellt. Als Anfangsbedingung habe ich neun Doppelschichten aus positiv geladenen Poly-L-Lysin (PLL) und negativ geladenen Hyaluronsäure (HA) gebildet. Eine solche Zusammensetzung von Polymeren erlaubt es, weiche Beschichtungen zu erzeugen welche schon als attraktives System für die Kultur der verschiedenen Zelltypen beschrieben wurden. Zudem können die mechanischen Eigenschaften solcher Schichten durch Versteifung mit einem kreuzvernetzenden Reagenz geändert werden, so dass halbsteife Substrate entstehen. Für die Herstellung von steifen Beschichtungen habe ich positiv geladene Poly-(Allylamin)-Hydrochlorid (PAH) und negativ geladene Poly-(Styrol)-sulfonat (PSS) verwendet. Dass solche Beschichtungen für die Bildung von Zellschichten geeignet sind wurde zuvor durch die Kultur von Endothelzellen gezeigt. Ich konnte darüber hinaus zeigen, dass die Steifigkeit der PEM Beschichtung eine entscheidende Rolle für die Adhäsion, die Ausbreitung und das Wachstum von menschlichen Zellen spielt. Obwohl der weiche Untergrund die Anhaftung der Maus Muskel-Zelllinie C2C12 unterstützt, konnte unter diesen Bedingungen nur eine minimale Haftung von menschlichen Zelltypen wie PD-MSCs, aus Fettgewebe gewonnenen MSCs (AT-MSCs), menschlichen Muskelzellen (HMC), menschliche Nabelschnur Endothelzellen (HUVECs) und menschliche Endothelzellen aus Nabelschnurblut „*Late Outgrowth Endothelial Cells*“ (OECs) beobachtet werden. Im Gegensatz dazu waren die halb-steifen und steifen Substrate für die Kultivierung von allen getesteten humanen Zelltypen geeignet und erlaubten die komplette mesodermale Differenzierung von AT-MSCs und PD-MSCs Zellschichten.

Im letzten Teil der Arbeit habe ich optimale Bedingungen für die Erzeugung und das Ablösen von PD-MSC Zellschichten etabliert. Um eine PEM-Architektur zu finden, welche die Zellschichten während des Wachstums minimal beeinträchtigt, entschied ich mich steife (PAH/PSS) Beschichtungen zu verwenden. Solche Beschichtungen unterstützten die Bildung von PD-MSC Zellschichten ohne chemische Versteifung. Außerdem konnten Zellschichten entweder durch Anwendung eines elektrochemischen Stimulus im Bereich von 1.8 mV oder durch eine saure Umgebung (lokaler pH 4) von der Oberfläche freigesetzt werden. Lebensfähige PD-MSC Zellschichten welche von (PAH/PSS) Substraten abgelöst wurden, konnten wieder auf Zellkulturschalen anhaften und dort in Richtung Adipogenese und Chondrogenese differenziert werden.

Abschließend kann festgehalten werden, dass PEM basierte Substrate sich als nützlich für die Kultivierung und Differenzierung von PD-MSCs erwiesen. Da diese Plattform das elektrochemisch und pH-vermittelte Schalen von Zellschichten ermöglicht, könnte sie in Zukunft für die Erzeugung homogener sowie gemusterten Zellschichten, welche zu gewebeähnlichen Konstrukten zusammengebaut werden, zum Einsatz kommen.

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## CHAPTER 1

# INTRODUCTION

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## 1.1. REGENERATIVE MEDICINE IN THE CONTEXT OF MODERN SOCIETY

---

Despite of the fact that the human body possesses the inherent ability to regenerate some specific cell populations and tissues, such as those of the liver, kidney, skin, and ribs, for instance, it is still very poor in terms of complete self-regeneration. In contrast to the majority of primitive species, high mammals and humans suffer from the inflammation and scar tissue formation that have replaced true regenerative abilities. For this reason, detriments such as damage, dysfunction, disease, or aging often lead to severe problems associated with morbidity, invalidity and mortality. Statistics show that annually, millions of patients worldwide require the structural and functional regeneration or replacement of organs and tissues.

The idea of generating live structures, from artificial tissues and organs to complex living organisms, was a matter of myths and dreams throughout the history of humankind. Starting from very early historical periods, legends concerning spontaneous wound healing or the creation of independent life without sexual reproduction, such as biblical tale about Eve emerging from Adam's rib or the myth about the regeneration of Prometheus's liver, illustrate an understanding of regenerative medicine from a naïve point of view [1]. However, parallel to such mythologies, the first efforts to reinstate the function of injured or lost body parts using non-living prosthetic devices (such as metal or ivory teeth and wooden limbs, for instance) were performed starting from antique times [2].

In early 1950's, as the concept of immunosuppression gained traction and relevant drugs became available, surgical transplantation became an *ultima ratio* for the problem of organ and tissue replacement. The combination of well-established surgical expertise with immunosuppression led to the rapid advancement of methods for solid organ transplantation. The first successful kidney transplant, between two identical twins, took place in 1954, followed by the first liver and lung transplant in 1963, and pancreas transplant in 1966 [3-5]. The first successful transplant of a human heart, in 1967, marked an important milestone in the era of mature, fully-functional organ transplantation [6]. The next quantum jump in transplantation occurred just one year later, with the first successful human bone marrow transplant in 1968 [7].

Yet, despite continuous development, the possibilities of surgical transplantation are still limited by its aging demographic base, increasing donor shortage, and associated post-operative complications. Modern transplantation is further impeded by the absence of ideal, artificial substitutes that can completely mimic native human organs and tissues. The development of new therapies for the treatment of aged, diseased, or damaged human tissues and organs is absolutely necessary [8-10].

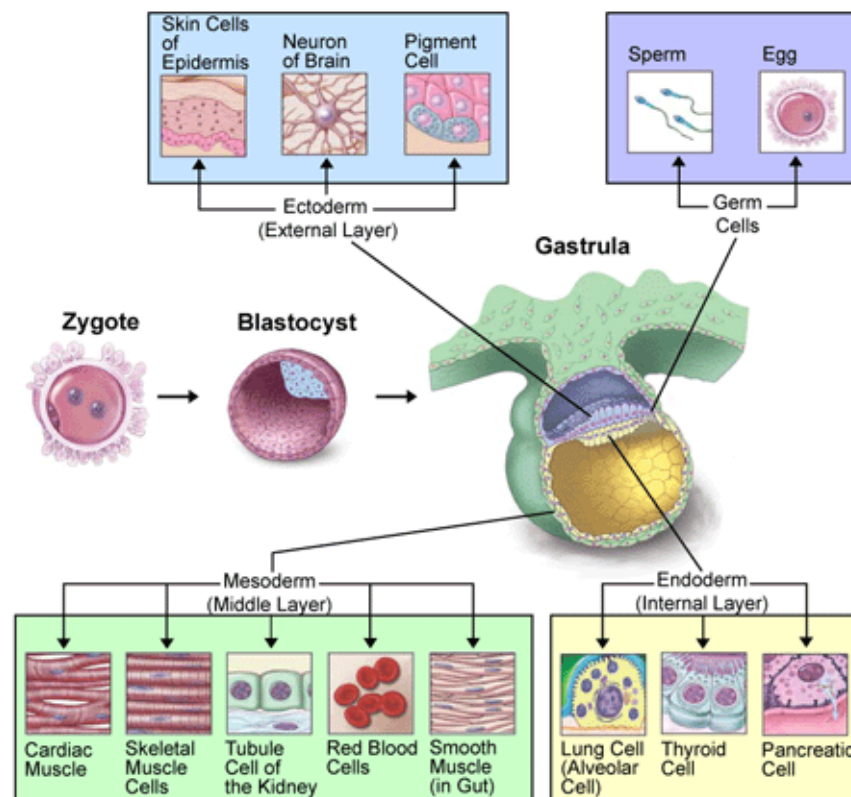
In the 1990's, the field of regenerative medicine, and tissue engineering in particular, became a promising alternative to traditional homologous transplantation [11]. Tissue engineering relies on the use of cellular and acellular components that either induce host tissue and organ regeneration or allow for the replacement of damaged tissue and organ by *de novo* - generated tissue substitutes. However, success in regenerating native tissues or their tissue-engineered substitutes depends on a thorough understanding of human organogenesis. Therefore, harnessing processes witnessed in both native organ formation as well as vascularization for artificially-induced tissue regeneration will provide the best chance of success in overcoming native organ dysfunction while establishing integration of the engineered substitute with the host vasculature [12].

## 1.2. HUMAN EMBRYONIC DEVELOPMENT AND ORGANOGENESIS

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Human evolution has resulted in the establishment of multicellular tissue structures and organs optimally adopted for specific functions in the body [13]. The formation of these highly organized and functionally integrated structures occurs mainly during organogenesis, the period of the intrauterine embryonic development. Organogenesis, starting from the fertilized oocyte (zygote), is characterized by a plethora of tightly coordinated events such as cell proliferation, differentiation, migration, and rearrangement. Immediately after its formation, the zygote undergoes a series of extremely rapid mitotic divisions wherein its cytoplasm is divided into numerous cells, forming a spheroid known as blastocyst. The inner cell mass (ICM) of the blastocyst consists of 70 to 100 pluripotent cells, also known as embryonic stem cells, that give rise to all differentiated somatic cell types in the adult body. These cells form the embryoblast as well as the outer trophoblast cell layer, which forms the placenta.

Reorganization of the blastocyst, ICM through gastrulation, leads to the formation of three definitive germ layers: the ectoderm, endoderm and mesoderm. From these layers, cells start to rearrange and develop the grounds of organs and complex tissue structures. Under the guidance of specific soluble molecules and growth factors, cells from each germ layer differentiate into a subset of specific cell types. The ectoderm gives rise to skin cells, neurons, pigment cells, the lens of the eye, epithelial structure of the mouth and anus, pituitary gland, and parts of the adrenal glands. The endoderm develops into cells of the lung, thyroid gland, thymus, pancreas, and liver, and additionally forms the lining of the gastrointestinal and respiratory tracts and bladder. The mesoderm develops into cardiac, skeletal, and smooth muscle cells, tubule cells of kidney, the urethra, gonads, bone, cartilage, and fat cells as well as bone marrow and red blood cells [Figure 1].



**Figure 1: Differentiation of three germ layers towards different tissue types. Picture adopted from [13].**

For the formation of functional tissues, cells derived from the different germ layers will have to reassemble in a highly specific arrangement. For example, in human skin, the epidermis is derived from ectoderm while the dermis is of mesodermal origin.

In summary, human organogenesis represents a complex cascade of closely connected physiological and anatomical processes leading to the subsequent development of specific cell types, tissues, organs, organ systems, and finally, the whole organism. Various stem cells, growth factors, and the extracellular matrix are key players in organogenesis [14].

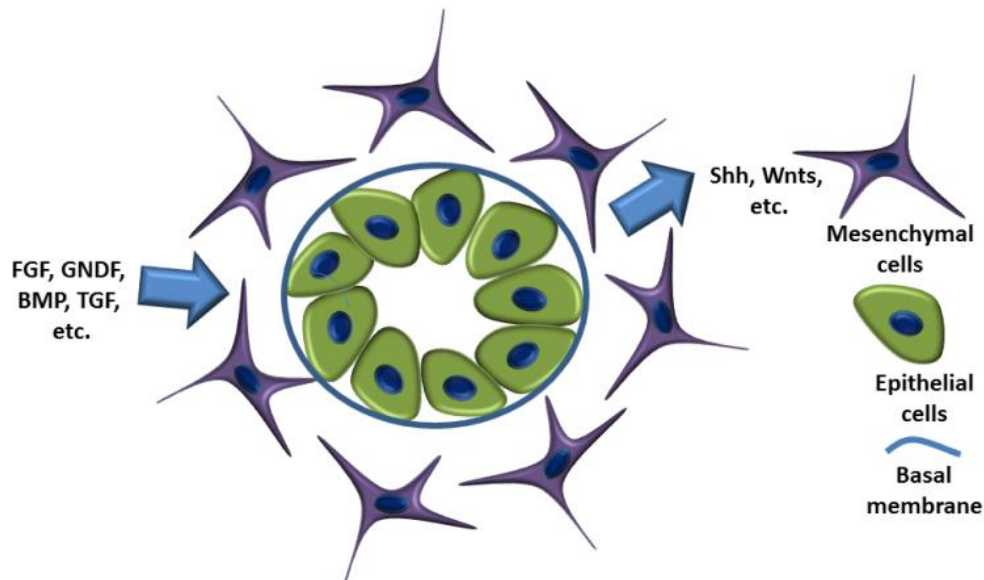
### 1.3. KEY FACTORS REGULATING ORGANOGENESIS

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The size of organs and of the whole organism closely related to the total cell mass, which depends on both the size and total number of cells. Organ and body size are determined by four continuous, cellular processes that all take place during organogenesis: proliferation, migration, differentiation, and apoptosis. During organogenesis, these processes are tightly regulated and controlled by numerous intracellular programs and extracellular signals. The extracellular signals that regulate cell size and cell number are normally soluble secreted proteins, proteins bound to the cell surface, or components of the extracellular matrix (ECM). They can be divided operationally into three main classes: mitogenes, growth factors, and survival factors [15]. In early development, some growth factors are provided to the embryo by the maternal system. In late embryonic development, growth factors are produced within the embryo itself [16, 17].

Mitogens are a specific group of more than fifty proteins that normally stimulate cell division during organogenesis. However, depending on environmental circumstances and the specific cell type, they can also be involved in cell growth, survival, migration, and differentiation. One of the first, most famous mitogens to be discovered is platelet-derived growth factor (PDGF). PDGF stimulates division of many cell types, including fibroblasts, smooth muscle cells, and neuroglia cells. Another mitogen, epidermal growth factor (EGF), acts on the division of epidermal, epithelial, and non-epithelial cells. Erythropoietin is an example of a marrow-specific mitogen, responsible for only inducing red blood cell precursor proliferation [15].

Like mitogens, extracellular growth factors are bound to cell surface receptors. These factors activate intracellular signalling, stimulating growth and increase in cell mass by promoting protein synthesis. Depending on the function, different types of growth factors exist. For instance, progression growth factors, such as endothelial growth factor (EGF) and insulin-like growth factor I (IGF-I), which guide the cells through the stages of cell cycle [18]. Inhibitory growth factors govern the tissue and organ enlargement. A representative example of this category is myostatin, a member of the transforming growth factor  $\beta$  (TGF- $\beta$ ) family that normally inhibits the proliferation and promotes fusion of myoblasts. Competitive growth factors are multifunctional secreted proteins with a wide variety of effects acting as both paracrine and autocrine signals. Competitive growth factors are responsible for molecular communications between different cell types, prevention of cell proliferation beyond excessive population density, and correct development of various tissues and organs [19]. For example, when interfaced with sonic hedgehog (SHH), competitive factors of fibroblast growth factor family, such as FGF7 and FGF10, regulate epithelial-mesenchymal interactions (EMI) [Figure 2].



**Figure 2: Molecular communications between different cell types during organogenesis. Picture modified from [1].**

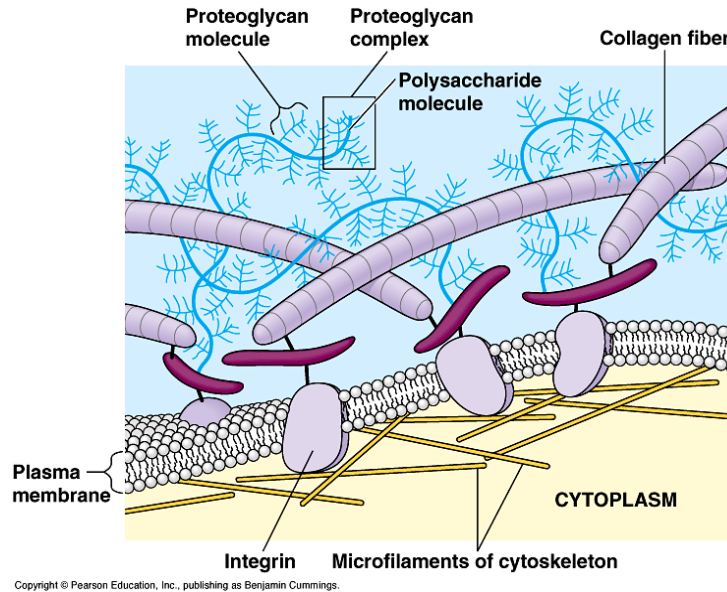
These EMI are critical for the development of epithelial-based structures such as seminal vesicle, salivary gland, and ventral prostate [20, 21]. Furthermore, FGF10 is also important for lung, thyroid, pituitary, pancreas, and lachrymal gland development

[22-26]. Survival factors are presumably responsible for the maintenance of the functional activities of stem cells. Bone morphogenic protein (BMP) is one of the survival growth factors. It usually binds cell-surface receptors and allows the cells to undergo pre-committed differentiation without inducing any real differentiation. BMPs promote cell survival by suppressing apoptosis [15]. However, specific genes and corresponding proteins that mediate survival are still poorly defined.

In summary, growth factors play an important role during organogenesis: they are responsible for the regulation of molecular mechanisms involved in the control of embryonic growth. Even a small perturbation of these growth factors can lead to the formation of diseased or abnormal fetuses. For example, shift in the production IGF-I can lead either to growth retardation or to macrosomia [27, 28].

As was mentioned above human tissues and organs represent multicellular arrangements with rather complex anatomical architecture. In order to generate functional tissues, the correct arrangement and organization of different cell types is necessary. The architecture of tissues is supported by the extracellular matrix (ECM), a cell-secreted compartment mainly composed of highly viscous proteoglycans damping the cells, fibrillar and non-fibrillar matrix proteins providing strength and elasticity, as well as soluble adhesive matrix-associated proteins binding the cells via integrin receptors [14]. Initially seen as an inactive scaffold, it became increasingly obvious that ECM components immobilized morphogenetic cues and provided important information to embedded cells [14]. Cells, on the other hand, continuously remodel the ECM such that the matrix composition changes over time **[Figure 3]**. During morphogenesis, when tissues and organ shapes are established, remodelling processes lead to the formation of instructive cues that guide the cell movement, rearrangement, and the formation of patterns. Therefore, the correct composition of ECM components (such as fibronectin, collagen, and laminin) and organ specific molecules (such as nephronectin) must be coupled with the expression of the corresponding receptors in order to incite correct tissue and organ development. The absence or imbalance of specific components and factors during ECM deposition or degradation will lead to malformation or failure in organ development [29].

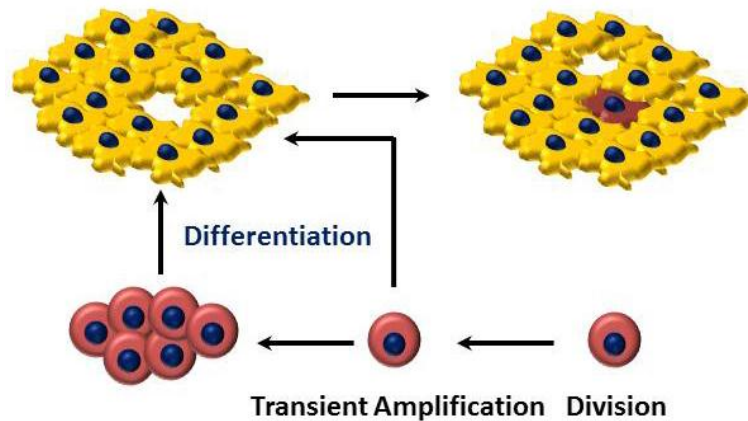




**Figure 3: Structure of extracellular matrix (ECM) by mammals. Picture adopted from [14].**

#### 1.4. POSTNATAL DEVELOPMENT AND HOMEOSTASIS

Embryonic development results in the complete formation of functional tissues and organs as well as determination of specific cell fate. Following the latter, cell proliferation leads to the growth of the whole organism through the enlarging and remodelling of tissues and organs. For enlargement of an organ, cell proliferation should be always accompanied by cell division, otherwise cells would get progressively smaller and total cell mass will not increase. Likewise with the process of organogenesis, control of cell growth and proliferation during the postnatal development is guided by extracellular growth factors and mitogens [15]. Finally, growth of the human organism is terminated during adolescence. However, despite completion of overall growth, any tissue injury, infection or necrosis in the organism can activate specific mechanisms of cell renewal called homeostasis. During homeostasis, constant cell production is required to sustain the replacement of damaged cells and tissue domains, thus balancing continuous cell death and maintaining native tissue [30]. Homeostasis represents a mechanism for the elimination of damaged or age-differentiated cells and their replacement by tissue-specific progenitor cells, referred to as adult stem cells [Figure 4].

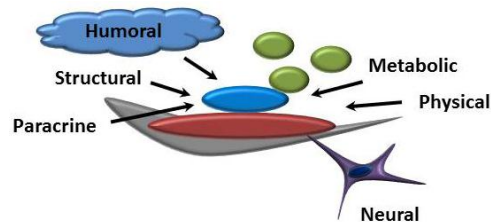


**Figure 4: Mechanism of cell turnover in solid organs. Picture modified from [31].**

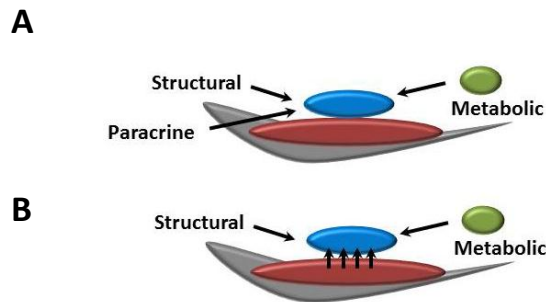
Adult stem cells are believed to reside in almost all tissues of the body and are thought to be localized in specific compartments called stem cell niches. Stem cell niches are described today as discrete and dynamic functional domains that are responsible for maintaining stem cells in their native status, replenishing the population of proliferative progenitor cells that give rise to differentiated cells, and governing tissue homeostasis under diverse physiological (development and aging) and pathological (injury and disease) conditions [32].

Architecture of the stem cell niches is only partially known. All stem cell niches represent defined anatomical compartments composed of the stem cell itself, micro-environmental stromal cells, and ECM proteins [32]. Additionally, the haematopoietic stem cell (HSC) niche contains blood vessels. All of these components play a defined role and are responsible for the correct function of the niche [32]. Micro-environmental stromal cells provide a specific barrier shield for the stem cells and defend them from signalling cues that would induce migration or differentiation. These stromal cells are also responsible for interactions between stem cells. The ECM proteins might provide mechanical and specific cell adhesion signals and support the structure of the niche. The blood vessels provide signals for the recruitment of circulating cells into the niche **[Figure 5]**. Anatomically, stem cells residing on the basal membrane of the niche are connected through homologous cell-cell interactions with micro-environmental cells, which build a regulatory environment **[Figure 6]**. Functionally, stem cell niches control the rate of stem cell proliferation and prevent excessive stem cell growth,

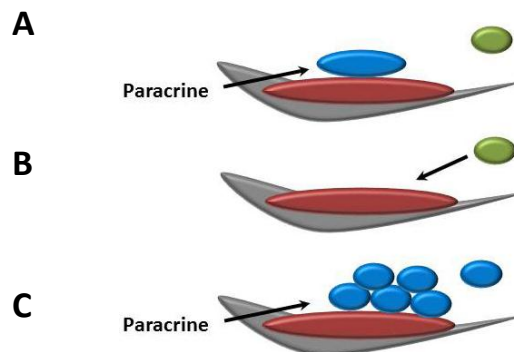
determine the fate of the stem cell daughter cells, and protect stem cells from death by exhaustion. Niches provide specific signals to their stem cells in form of the intrinsic secreted factors or surface molecules [Figure 7].



**Figure 5: Hypothetical structure of the stem cell niche. Picture modified from [32].**



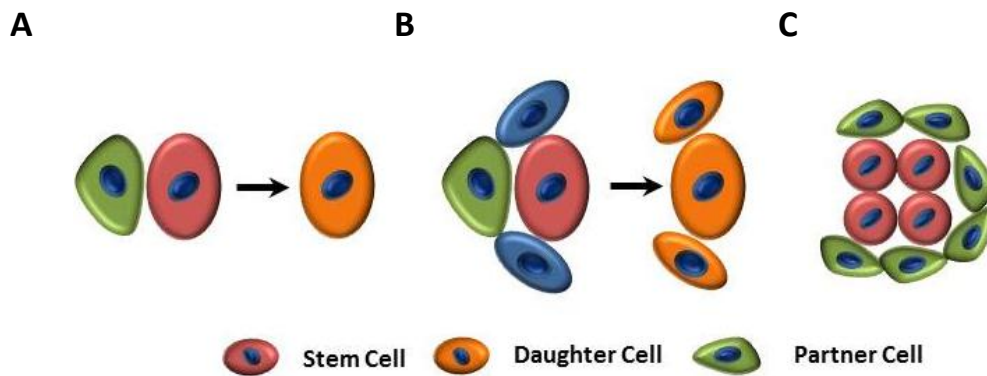
**Figure 6: Residing of stem cell in the niche. (A) Three dimensional structure of the niche. (B) Regulatory microenvironment of the niche. Picture modified from [32].**



**Figure 7: Supposed mechanism of niche contribution to dysplastic cell growth. (A) Normal occupation of stem cell niche, (B) Ectopic occupation by another cell type in case of the vacancy in the niche, (C) Disordered state of tissue organization and control improved by proliferative and de- differentiative signals. Picture modified from [32].**

Stem cell niches do not always incorporate the aforementioned components. Generally, they represent various entities containing different combinations of niche components and multiple inputs, all of which are established for accomplishing of the exquisite control for particular type of stem cells involved in homeostasis.

Depending on the specific function, stem cell niches can be divided into three sub-types: simple cell niches, complex cell niches, and storage niches [Figure 8]. The simple cell niche represents a micro-environment in which one stem cell type is locked through adhesion junctions and integrin-mediated interactions to the extracellular matrix. In this niche, stem cells receive paracrine signals that promote their growth while inhibiting differentiation. The complex niche contains different stem cell types at once. The storage niche contains specific resident stem cells that activate in case of trauma [33]. These cells subsequently divide and migrate to repair injured tissue.



**Figure 8: Proposed types of the cell niches. Simple niche (A), complex niche (B), and storage niche (C). Picture modified from [33].**

Only strongly-balanced interactions between all components sharing the niche ensures the maintenance of the stem cell phenotype and guides differentiating daughter cells away from the niche [34]. After leaving the niche, stem cells are no longer controlled by intrinsic and extrinsic niche derived factors that maintain their undifferentiated phenotype. As a result, they start to differentiate, first into transiently amplifying progenitor cells, followed by continuously amplifying progenitor cells. Depending on morphogenetic cues, progenitor cells will further differentiate along a specific lineage until achieving a terminally differentiated adult phenotype. At this stage, terminally

differentiated cells execute specialized functions and their proliferative potential is restricted to a few cycles.

Depending on the homeostatic situation, niches can appear for the recruitment of stem cells, which are necessary for the particular anatomic location in a concrete tissue or organ. Once formed, niches are responsible for local regenerative cues and interactions with supporting stem cells. Three different mechanisms for specific stem cell niche establishment have been proposed. The first mechanism is associated with embryogenesis. In this case, the niches are built from heterologous cell types during the time of organ formation and remain stable during growth, adolescence, and homeostatic periods independent of stem cell presence during their occupation [32]. The second mechanism might be described as post-embryonic co-development of stem cells, with micro-environmental cells with following formation of the niches because of their close localization and continuous interactions [32]. The third mechanism is based on the ability of some cell types, such as hematopoietic stem cells (HSCs) or primordial germ cells (PGCs), for instance, to form stem cell niches during proliferation. In this case, organisms could customize different niches in order to support symmetric or asymmetric division, facilitate rapid proliferation, impose stem cell quiescence, and bias differentiation of progenitor cells towards specific lineage [35].

Despite to the functional diversity between stem cell niches, their key components remain the same across different tissues and organs. In all cases, the niche provides structural and trophic support as well as topographical information and the appropriate physiological cues, all of which are necessary for the regulation of stem cell function [35].

The exact number of specific niches that harbour stem cells and maintain continuous cell turnover during life cycle is still unknown. However, at least three types of stem cell niches, which actively contribute to homeostasis, are becoming elucidated [36]: the niche of intestine, known as the interstitial stem cell niche (ISCN) [36], the hair follicle epidermal stem cell niche (HFSCN) [37, 38], and the hematopoietic stem cell niche of the bone marrow (HSCN) [39]. All of these stem cell niches share the common properties described above [36]. At the same time, intricate signalling of interstitial and follicle epidermal niches is dependent on the surrounding mesenchymal cells [36, 40].

The MSC niche itself also plays a very important role in homeostasis [35, 36, 41]. Based on the fact that MSCs isolated from the vast of postnatal tissue types expressed alpha smooth muscle actin ( $\alpha$ -SMA) and pericytes-associated cell-surface marker 3G5, a perivascular nature of MSC niche was hypothesized [42]. According to the perivascular theory, MSCs localized in the perivascular niche are distributed throughout the body and can easily access to all tissues in case of injury. However, *in vivo* experimentation is still required to test this theory.

Taken together, human tissue homeostasis represents a dynamic equilibrium which maintains an internal *status quo* within a defined tissue of an organism through the continuous regulation of cellular proliferation and death as well as control of metabolic function. A misbalance between cell renewal and cell death during homeostasis can lead to disease or even death of the individual. A stem cell niche is a micro-environmental unit to maintain stem cells and to replenish the pool of progenitor cells during the homeostatic regeneration of tissues and organs. They are thought to be involved in the process of tightly regulating the self-renewal of the adult stem cells. Recent investigations show that different niches are adopted to their unique function and even small misbalance between structural parts of the niche can lead to pathological degeneration of tissue, aging, decrease of regenerative potential, and malignancy [35]. For example, if the production of liver cells outweighed their death by only 1%, liver weight would be equal to initial body weight within a period of 6 to 8 years [33].

## 1.5. ROLE OF MESENCHYMAL STEM CELLS IN TISSUE HOMEOSTASIS

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Post-natal tissues of the human body harbour different numbers of progenitor cells, all of which can give rise to cell types of the specific tissue in which they reside. Such cells were first isolated from human bone marrow (BM) and described as marrow stromal cells (MSCs) in 1970's [43, 44]. Recently, cells with similar properties were isolated from adipose tissue, epidermis, liver, pancreas, brain, dental pulp, spinal cord, etc. [45, 46]. Unlike the totipotent embryonic stem cells (ESCs) which are responsible for embryonic development and foetal growth of an organism, multipotent adult MSCs contribute to homeostasis as well as to regeneration of diseased organs and damaged tissues. Moreover, during the process of tissue homeostasis, MSCs are also involved in the

processes of self-tolerance and affect the immune system, which is critically important for tissue regeneration [47]. The exact origin and primary location of MSCs participating in tissue homeostasis still remains debatable and unclear.

Three different hypotheses exist with regards to the specific location of these cells in an organism. The first hypothesis suggests that MSCs are located in only a few specific tissues or organs. Upon homeostatic demand, they would exit their sedentary locations and migrate to other tissues and organs in order to replenish the apoptotic adult cell populations in case of physiological turnover or pathological necrosis [47]. The second hypothesis is based on the suggestion that MSCs with very similar immuno-phenotypic and functional properties reside in all postnatal tissues of the body, especially in the form of different tissue-intrinsic progenitor cells that can be recruited during regeneration [47]. The third hypothesis suggests that MSCs reside in a perivascular site. In case of disruption of blood vessels or neighbouring tissues, tissue specific progenitors can be liberated in form of pericytes-like cells, which subsequently migrate to the site of need, proliferate, and secrete bioactive factors in order to protect and regenerate the specific tissue [47]. Based on this hypothesis it has been speculated that pericytes could be an *in vivo* source of mesenchymal cells for *in vitro* applications [48]. One argument that supports this view is that pericytes can give a rise to adult cell types such as adipocytes [49], chondrocytes [50], and osteoblasts [51].

The main criteria that distinguish adult MSCs from end-differentiated, adult cell types are their specific stemness properties (properties that confer the ability to self-renew and to differentiate into cells of different lineages). Stemness include: the ability for self-renewal, plasticity or differentiation potential, and regenerative potential.

Self-renewal is the ability of stem cells to produce daughter cells with identical stem cell features as the mother cell. Stem cell division can take place either symmetrically or asymmetrically. In case of symmetric division, both daughter cells have the same fate and become new stem cells. In the case of asymmetric division, one daughter cell becomes a new stem cell while another one will differentiate into a specific cell type. Whether the balance between symmetrical and asymmetrical divisions or the number of divisions is responsible for the generation of appropriate amounts of stem cells and differentiated cells in an organism at this stage remains unclear. Many different

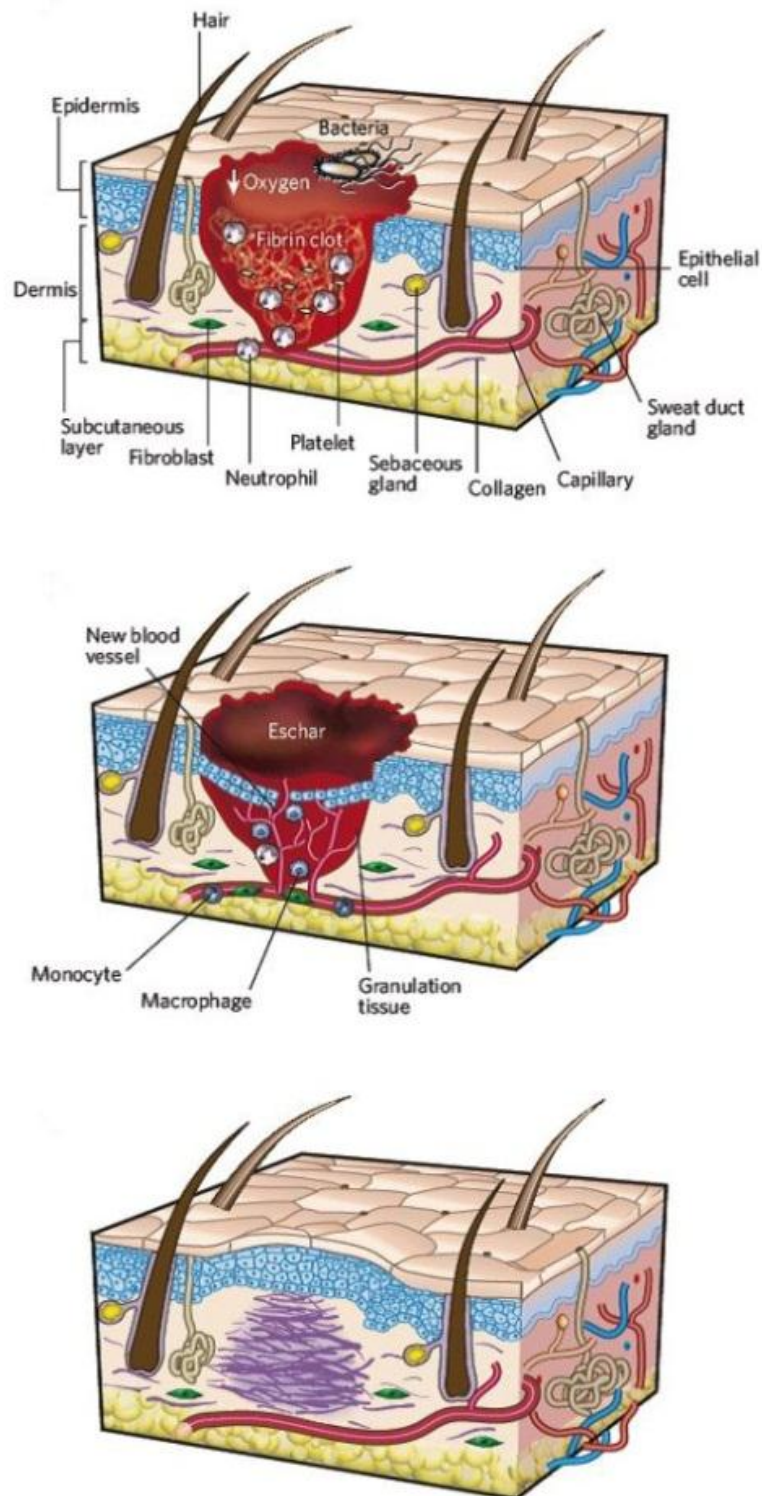
pathways might be involved in stem cell self-renewal and some of these are well described, but others are still debatable or unknown. Self-renewal regulation can be coordinated by cell cycle and proliferation. Curiously, the same pathway mechanism can stimulate differentiation of one cell type while being involved in the maintenance of self-renewal potential by other cell types. For example, the process of stem cell renewal can be regulated through the chromatin structure by polycomb group proteins (PcG), which represses the transcription of genes that regulate differentiation. Examples include Bmi-1, Mel-18, and Rae-28. In another regard, depending on the specific situation, self-renewal can be also involved such signalling pathways as Notch, Wnt, and Hedgehog. Differentiation potential or plasticity is a property in which stem cells become restricted in their capacity to form a variety of different cell types; rather, they specialize to form more specific cell types that fulfil particular, focused functions within the body. The spectrum of cell types, towards which adult stem cells can differentiate, is determined by their potency. Adult stem cells, which can form only one particular cell type within one particular lineage, are unipotent. Tissue specific MSC-like cells are multipotent and can be differentiated into multiple specialized lineages, such as adipogenic, chondrogenic, osteogenic, or myogenic, for instance. Adult stem cells can be differentiated towards a broad spectrum of adult cell types within one particular lineage, such as neural stem cells or hematopoietic stem cells, and are referred to as oligopotent stem cells.

## 1.6. TISSUE REGENERATION

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Tissue regeneration, in contrast to the genetically programmed, slow-cycling process of homeostasis, involves the secretion of exogenous factors that cause acute damage and can provoke a strong inflammatory response – wounding [31]. Tissue repair consists of several steps **[Figure 9]**. Immediately following injury, the blood coagulation cascade is activated by platelet tissue factor called thrombokinase. The blood clot is normally formed within several minutes by aggregation of fibrin and thrombocytes. At the same time, blood vessels surrounding the injury site are constricted in order to reduce haemorrhage. Stimulated by platelet-released factors, vasodilatation and vascular permeability are induced in neighbouring blood vessels.





**Figure 9: Stages of tissue regeneration process: (A) Inflammation, (B) New tissue formation, (C) Tissue remodelling and scarification. Picture adopted from [52].**

The activation of the clotting cascade leads to the formation of a fibrin plug, which, in combination with fibronectin, serves as provisional matrix for the subsequent recruitment of inflammatory cells and migration of tissue resident MSCs. At the second stage, inflammatory cells, such as neutrophils and macrophages, are recruited through release of the mitogenic and chemotactic factors, such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- $\beta$ ), and tumour necrosis factor alpha (TNF- $\alpha$ ). After the destruction of pathogenic microorganisms by inflammatory cells, surrounding endothelial cells start to form new blood vessels. At the third stage, tissue resident MSCs migrate towards the site of injury and together with macrophages and fibroblasts replace the provisional matrix with granulation tissue. In the fourth stage the wound becomes contracted. [47]. The final stage of normal wound healing process is formation of the scar, which appears through the stimulation of collagen synthesis [52, 53]. The mechanisms involved in MSC migration during tissue regeneration are still debatable. A first hypothesis suggests that specific receptors, facilitating trafficking of MSCs and regulating their following adhesion and infiltration, are located in the MSC niche. They are immediately activated after injury through the release of factors, such as stem cell factor (SCF) or stromal-derived factor 1 (SDF-1). A second hypothesis proposes that integrins, selectins, and chemokine receptors expressed on the surface of MSCs, are responsible for MSC migration to the injury site. The third hypothesis is based on the assumption that MSCs passively reside in capillaries and micro-vessels directly interact with accessory pericytes, thus recruiting MSCs to the injury site through release of soluble growth factors and trophic cytokines [54-56]. The latest studies also suggest that MSCs secrete trophic, immunosuppressive and antifibrotic factors which minimize the extent of damage and lead to the reduction of inflammatory response of the organism. The capacity for self-regeneration in humans is directly connected with human complexity. Depending on parameters such as the age of an individual, type of tissue, accompanying diseases, or genetic disposition, the efficiency of regeneration can vary drastically. The regenerative potential of all human tissues and organs is strongly dependent on the rate of cell turnover and is regulated through the contribution of the tissue specific (MSCs) and hematopoietic (HSCs) stem cells [57]. According to their

regenerative potential, all human tissues and organs can be divided into three categories [Figure 10].

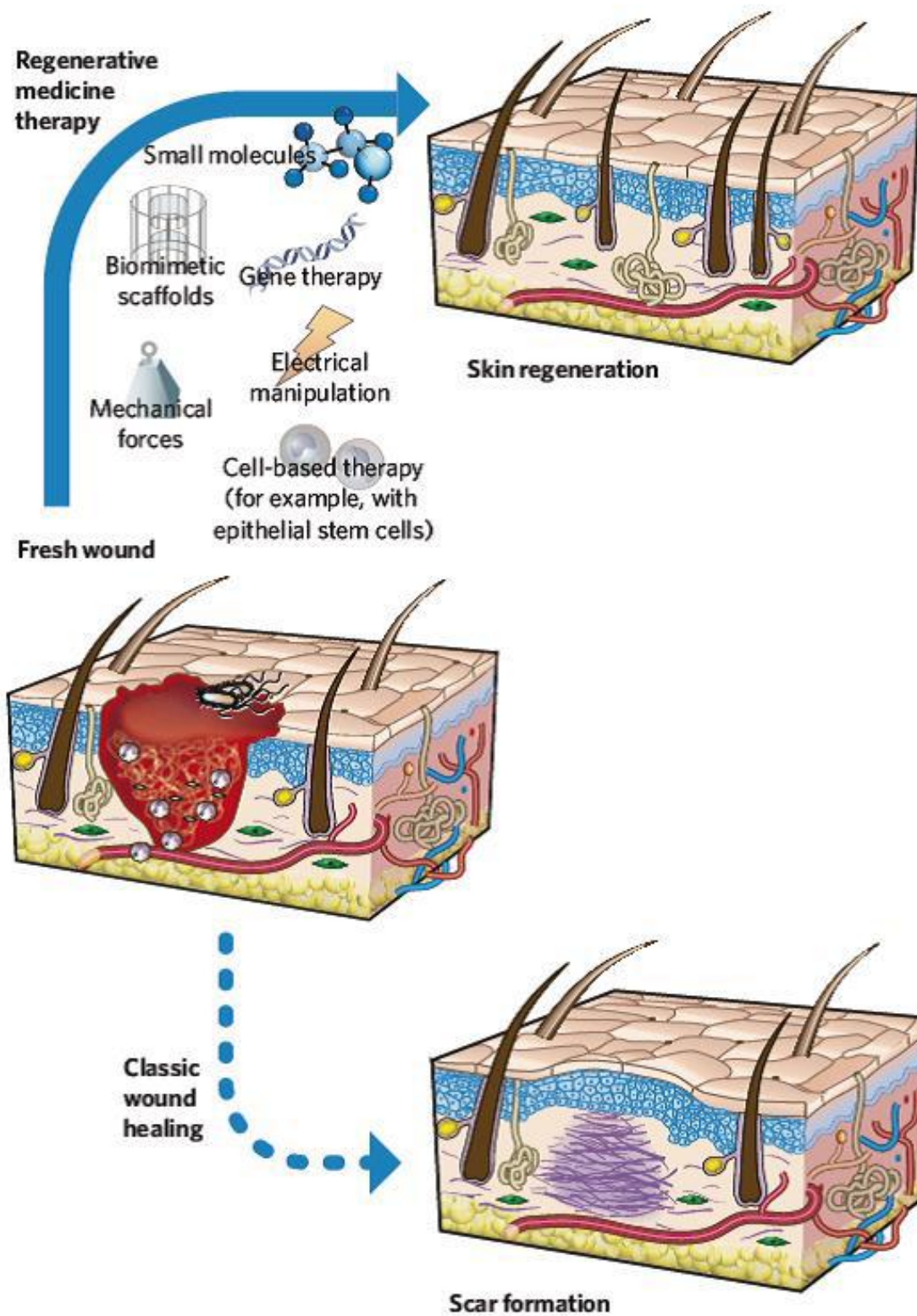


Figure 10: Contribution of stem cells to homeostasis. Picture modified from [57]

The first category contains organs and tissues that possess a high cell turnover, a high regenerative capacity, and a prominent stem cell compartment such as blood, skin, and gut. The second category is comprised of organs and tissues with low cell turnover and high regenerative potential. An example lies with skeletal muscles, because they are unable to proliferate and generate new tissue, but can recruit resident stem cells for turnover and regeneration. Organs of the third category, such as the brain and heart, show low endogenous repair capacity, low cell turnover and low regenerative potential.

The most famous example of self-regeneration in human organs lies with the liver. The human liver is a unique organ which can regenerate its whole mass from any remaining fragments smaller than 25% of the native organ mass [58]. This ability is due to the hepatocyte capacity for self-renewal. Resection of the liver induces proliferation of remaining hepatocytes until the whole mass of the organ is restored [59]. Interestingly, the liver never overgrows its original size; instead, it is adapted to the size of an organism as demonstrated by transplantations of liver lobes into children. This process is thought to be regulated by the circulating homeostatic factor, the hepatocytes growth factor. In addition to the liver, the fingertips, ribs, and kidney are other examples of human tissues and organs with the potential for self-regeneration [60-62].

The regeneration of the damaged tissues and organs induced by the organism itself is one of the most complex and uncontrollable biological processes that takes place during human life. However, even though the human body possesses an inherent ability for homeostatic cell turnover as well as regeneration of selected cell and tissue types, healing might often be delayed or excessive. Delayed healing processes normally lead to the formation of chronic wounds, while excessive healing processes are related with formation of non-functional fibrotic tissue. Due to these issues, the inherent regenerative potential of an organism might be insufficient for complete restoration of the organ or tissue function in case of pathological disease and trauma. Traditional surgical methods of organ and tissue transplantation as well as use of biomechanical devices and artificial organ-like structures are also imperfect solutions for the restoration or support of damaged organs and tissues. Thus, the establishment of new strategies for the transformation of fibrotic healing processes towards functional tissue regeneration are absolutely necessary **[Figure 11]**.



**Figure 11: Advantages of artificial organogenesis comparable with tissue regeneration.**  
**Picture adopted from [52].**



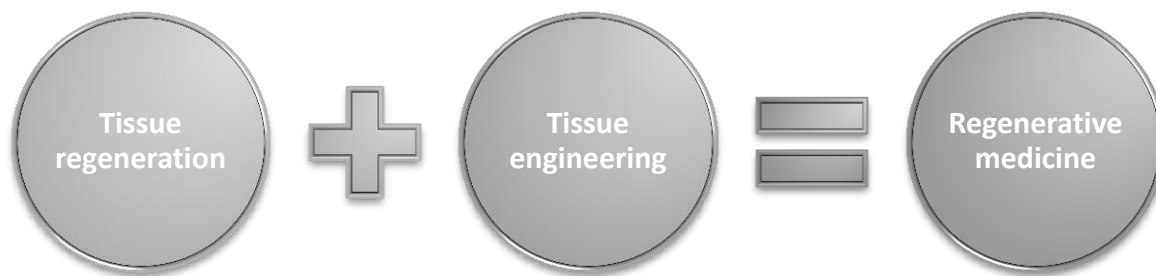
## 1.7. REGENERATIVE MEDICINE

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Regenerative medicine is an extended field of medicine that aims to induce healing for the replacement of defective tissues and organs. The term “tissue engineering” was classically used in the context of surgical manipulations and the application of prosthetic devices or biomaterials for tissue or organ reconstruction. In 1987, Langer and the Vacanti brothers introduced a modern definition of tissue engineering: “Tissue engineering is multidisciplinary field combining principles of life science with methods of engineering for the development of functional biological substitutes that restore, maintain or improve tissue and organ function” [11, 63]

On one hand, a modern definition of regenerative medicine states that the ultimate goal is “to replace living functional tissue with living functional tissue that is designed and constructed to meet the needs of each individual patient” [64, 65]. On another hand, the final goal of regenerative medicine can be described as implementing a functional and structural restoration as well as *de novo* engineering and substitution of diseased or damaged human tissues and organs.

The main approaches of regenerative medicine were introduced almost two decades ago and are based on two different principles: tissue regeneration and tissue engineering. Dependent on specific clinical demand, these approaches can be realised using many specific strategies and methods which have some advantages and some disadvantages [Figure 12].



**Figure 12: Main approaches of regenerative medicine.**

Most useful strategies and methods of regenerative medicine include the transplantation of acellular substances and biomaterials that promote tissue regeneration, infusion of purified and mixed cell suspensions, and transplantation of

bioengineered structures which represent combinations of specific cell types and materials [64-66].

## 1.8. STRATEGIES FOR TISSUE REGENERATION

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Tissue regeneration is mainly based on the repair of damaged host tissues by inducing the tissue's own regenerative potential. The most popular strategies include the infusion of purified or mixed cell suspensions, transplantation of acellular substances inducing tissue regeneration, and transplantation of developing organs.

The direct transplantation of isolated cells is based on the assumption that cells can directly integrate into tissues and support the regenerative processes if they are placed into specific microenvironment. Typically, direct cell transplantations are performed either topically or systematically through the injection of autologous or allogeneic cell suspensions. Such procedures avoid complicated surgical interventions and permit different cell manipulations before injection [67]. The disadvantages of direct cell transplantation include low tissue integration and high death rate of injected cells as well as little control over cell functions after transplantation. Furthermore, due to the high risk of post-transplant complications, such as associated infections, veno-occlusive disease, mucositis, graft-versus-host disease (GVHD), and graft-versus-tumour effect (GVT), this procedure is traditionally reserved for patients with life-threatening diseases [11, 64].

Transplantation of tissue-inducing substances is based on the delivery of specific signal molecules, such as growth factors, to the damaged organ or tissues in order to stimulate organ/tissue self-regeneration. On one hand, this method is minimally invasive and easily controllable, while on another hand, its efficiency is strongly dependent on the purification and large-scale production of appropriate growth factors as well as development of specific delivery strategies, which will provide tissue inducing agents precisely to the targeting site [68].

Transplantation of entire or partial developing embryonic organs into adult recipients is known as the *intracorporeal* tissue regeneration approach. In contrast to strategies that employ initially desegregated donor cells, cells in transplanted developing organs remain in their natural microenvironment and can thus continue to fulfil their

physiological function. This method has been experimentally established for the reconstruction of kidney. Due to the avascular nature of renal tissue during the initial stages of development, this organ possesses very low potential of immuno-rejection [69]. Results showed that parts of embryonic kidneys as well as whole organs could be successfully implanted into the adult host [70, 71]. However, due to the serious ethical and practical problems associated with the use of material derived from human embryos, the application of embryonic organ rudiments is not a clinical option at the moment [1].

## 1.9. STRATEGIES FOR TISSUE ENGINEERING

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The tissue engineering approach is based on the replacement of damaged host tissues by *de novo*-generated, functional, tissue-like substitutes. Classical tissue substitutes can arise from cells, biomaterials or their combinations. Such substitutes should integrate into the patient as soon as possible and be allowed to immediately begin restoration of specific tissue or organ function [11, 63, 72]. Tissue-engineered substitutes can be generated *ex vivo* outside of the body (*extracorporeally*) or *in situ* inside of the body (*intracorporeally*). Unlike *ex vivo* tissue engineering, where the whole process of tissue formation and partially also tissue remodelling occurs outside the body, *in situ* tissue engineering initiates and completes the generation of tissue substitute inside the body.

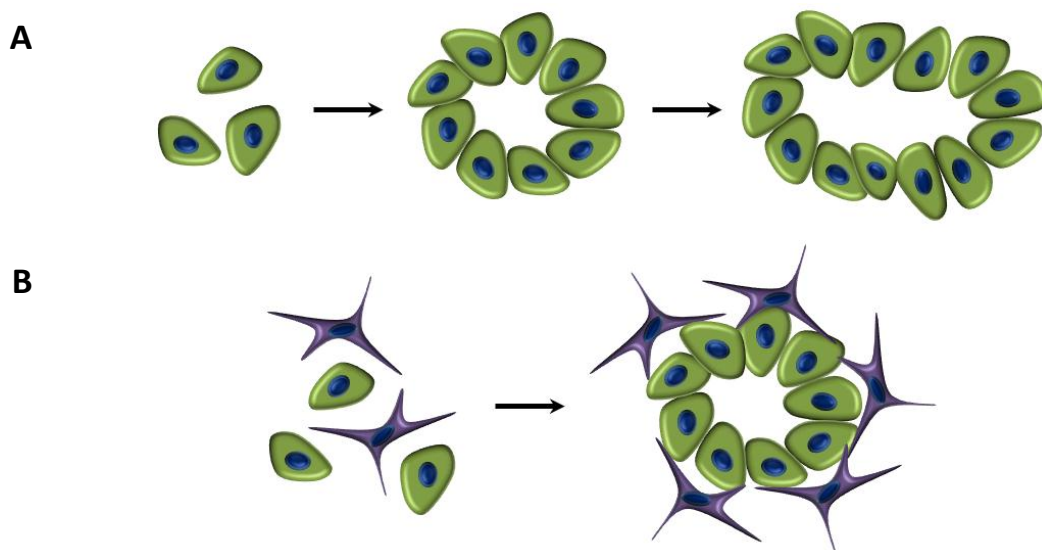
Initially, *ex vivo* tissue engineering strategies were limited to simple tissue substitutes fabricated under static cell culture conditions *in vitro* before implantation. Today, dynamic devices, such as flow-through systems and complex bioreactors are employed to generate functional tissue substitutes prior to the transplantation. In order to form such constructs, *in vitro* expanded cells are seeded into 3D scaffolds that might provide the proper physicochemical and biological cues to support cell proliferation and differentiation. The cell-seeded scaffolds are incubated in a bioreactor with specific conditions (flow) to stimulate the correct development and growth of the tissue substitute. Once the cellular construct has consolidated its structure and reached the desired functionality, the *ex vivo* generated tissue substitute will be removed from the bioreactor and subsequently transplanted into the living organism. Although, two *ex vivo* generated scaffold-based tissue substitutes, tissue engineered heart valves and



blood vessels, are now reaching late pre-clinical testing [73, 74], major limitations restrict the use of *ex vivo* tissue engineering strategies for other applications. The diffusion of nutrients and waste products as well as limited gas exchange does not allow for the fabrication of large tissue substitutes and complex artificial organs. Furthermore, the preservation of viability and functionality of *ex vivo* generated tissues post transplantation requires a preformed functional vascular system. Finally, the inflammatory response towards biomaterial remnants might inhibit the successful integration of the transplant.

In situ tissue engineering is based on the general idea that the human body is the best bioreactor, providing ideal conditions for tissue development and regeneration. The four most prevalent approaches of intracorporeal tissue engineering include: transplantation of individual cells in form of purified cell suspensions, transplantation of acellular inducing substances stimulating regenerative processes of the target tissue, transplantation of developing organs, and transplantation of cells attached to the biocompatible scaffolds [66].

The concept of scaffold-based tissue engineering corresponds to physiological tissue remodelling and morphogenesis [Figure 13].



**Figure 13: Spontaneous remodelling of embryonic cell cultures cultured *in vitro*. (A) Organization of polarized cyst by foetal epithelial cells cultured in 3D matrices. (B) Organization of epithelial and mesenchymal compartments by mixtures of foetal lung cells cultured in 3D matrices. Picture modified from [1].**

Under appropriate environmental conditions dissociated cells can reassemble *in vitro* into structures that resemble the native tissue [75]. Furthermore, cells in native tissues are adherent and located within the cell instructive three-dimensional viscoelastic ECM. In this milieu matrix, associated signalling cues and a three-dimensional arrangement of cells are provided. These cell-matrix and cell-cell interactions play an important role in tissue development, maintenance and homeostasis, as well as tissue regeneration [76].

The use of matrices, which mimic some of the signalling properties of the ECM and provide mechanical support for *de novo* formation and structural stabilization of engineered tissues, can be considered as classical algorithm of tissue engineering [77, 78]. Technically, scaffold-based tissue engineering is performed by placing cells on or within bio-instructive scaffold followed by transplanting this cell-scaffold construct into the recipient. Until recently, it was not possible to correctly mimic the anatomical structure and physiological functions of ECM for guiding morphogenesis. Novel technological platforms that allow for both proper positioning of cells and matrix components as well as concurrently defining stem cell fate will make controlled, guided morphogenesis possible. [78]. Initial attempts to form meaningful artificial structures consist of spotting and printing cells and materials in 3D architectures [79].

Scaffold-free tissue engineering represents an alternative to scaffold-based tissue engineering. It is based on the assumption that absence of any biomaterial compartment increases the potential of tissue-like structures for correct integration into the body and decreases the risk of infections as well as complications related to the foreign body reaction. Micro-tissues are an excellent example of a scaffold-free tissue engineered structure. Micro-tissues (also termed organoids) represent functional cell aggregates sized between 100 and 500 nm in diameter [80]. They are generated from either enzymatically or mechanically dissociated tissues [81, 82], cell lines [83, 84], or stem cells [85]. Experimental results have shown that micro-tissues can be assembled from various cell types such as cardiomyocytes, neurons and chondrocytes [86-88]. Organoids have many advantageous properties that make them potentially attractive for future clinical use. Due to their relatively large size and extensive ECM, micro-tissues are mainly retained at the site of application and have a good grafting potential. Moreover, emerging evidence has demonstrated that micro-tissues produce

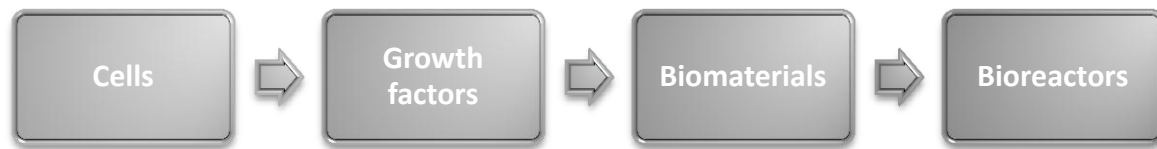
proangiogenic factors, such as VEGF, that potentially support pre-vascularization *in vitro* or induce angiogenesis after implantation [89-91]. Additionally, organoids can be used as building blocks for assembling of scaffold-free tissue-like structures, which can be produced in moulds of defined shapes. Experiments in animals demonstrated that tissue-like patches assembled from single human chondrocyte-composed micro-tissues are able to produce a sufficient level of collagen II and adapt to native-like cartilage morphology [90]. Despite this promising potential, the micro-tissue approach still remains inadequate for direct clinical applications because of difficulties related with blood supply and controlling of organoid shape after implantation [92, 93].

Though regenerative medicine is relatively young compared to other medical disciplines, the first successful attempts in the field were demonstrated in the 1970's by Dr. John Burke and Dr. Iannas Yannos. They generated *de novo* skin substitutes using collagen matrix-supported growth of dermal fibroblasts. At the same time, Dr. Howard Green successfully treated burn patients by transplanting sheets of keratinocytes. The clinical successes of these efforts as well as their commercial potential led to the new concept of extracorporeal tissue generation *in vitro* and, in the 1990's, to the revolutionary idea of manufacturing "off-the-shelf" tissue or organ replacements. This was a driving force for the organization of the first tissue engineering programs, non-commercial initiatives, and commercial companies with the primary aim of overcoming the main problems of traditional transplantation [94].

## 1.10. MAIN PILLARS OF TISSUE ENGINEERING

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For proper patho-physiological regeneration *in situ* or the *ex vivo* engineering of functional tissue, a careful selection of specific cell types, biomaterials, metabolic growth factors, and biomechanical environmental conditions is crucial. Today's tissue engineering efforts combine the latest achievements of stem cell research, materials science, and nanotechnologies as well as biophysics and bioinformatics. During the last two decades, tissue engineering has made tremendous progress. The main pillars for the successful development of functional complex tissue and organ substitutes were established [Figure 14].



**Figure 14: Main pillars for successful development of complex tissue and organ substitutes**

Based on their physiological role *in vivo*, cells are often considered to be the building blocks for artificially-induced tissue regeneration. Functionally, cells should have the capacity to produce missing tissue components and restore the function of the host tissue and organ. For this reason, choosing the appropriate cell type is critical for the success of any cell-based therapy.

Soluble growth factors include cytokines, growth factors, morphogenetic proteins, small molecule agonists, steroid hormones and ions. These factors feed into complex, interacting networks of signal transduction pathways and regulate cell growth, differentiation, and apoptosis. Refer to Chapter 1.4 for more details.

According to Williams' 1987 definition of biomaterials, biomaterials are nonviable materials used in a medical device and intended for interactions with biological systems [95]. Biomaterials in tissue engineering serve as structural supports or cell adhesion sites, and can be engineered for biologically-active molecule delivery. Moreover, supporting structures engineered from biocompatible materials can promote viability, expansion and incorporation of transplanted cells. Biomaterial properties influence the formation of structurally and functionally different products, such as hydrogels or porous scaffolds. Biomaterials are very diverse, ranging from hard materials (such as Calcium Phosphates) to soft naturally-derived polymers (such as fibrin and collagen) as well as fully-synthetic polymers (such as polyethylene glycol).

Bioreactors are closed, fully controllable systems that play an integral role in the cultivation and expansion of cells for *extracorporeal* tissue and organ generation. Bioreactors allow for continuous observation and manipulation during the whole process of tissue substitute formation. A tight control over flow, mixing regimen, and medium composition, including application of defined amounts of bioactive molecules, can be achieved within the bioreactor. Moreover, if needed, bioreactors can hydro-

dynamically stimulate cells and thereby increase the production of glycosaminoglycans or collagens leading to biomechanical stabilization tissue substitutes [64, 96].

Depending on the design and type of working system, different bioreactors exist: hollow-fibre, perfusion, stirred tank, and rotary.

### 1.11. CELL SOURCES FOR TISSUE ENGINEERING

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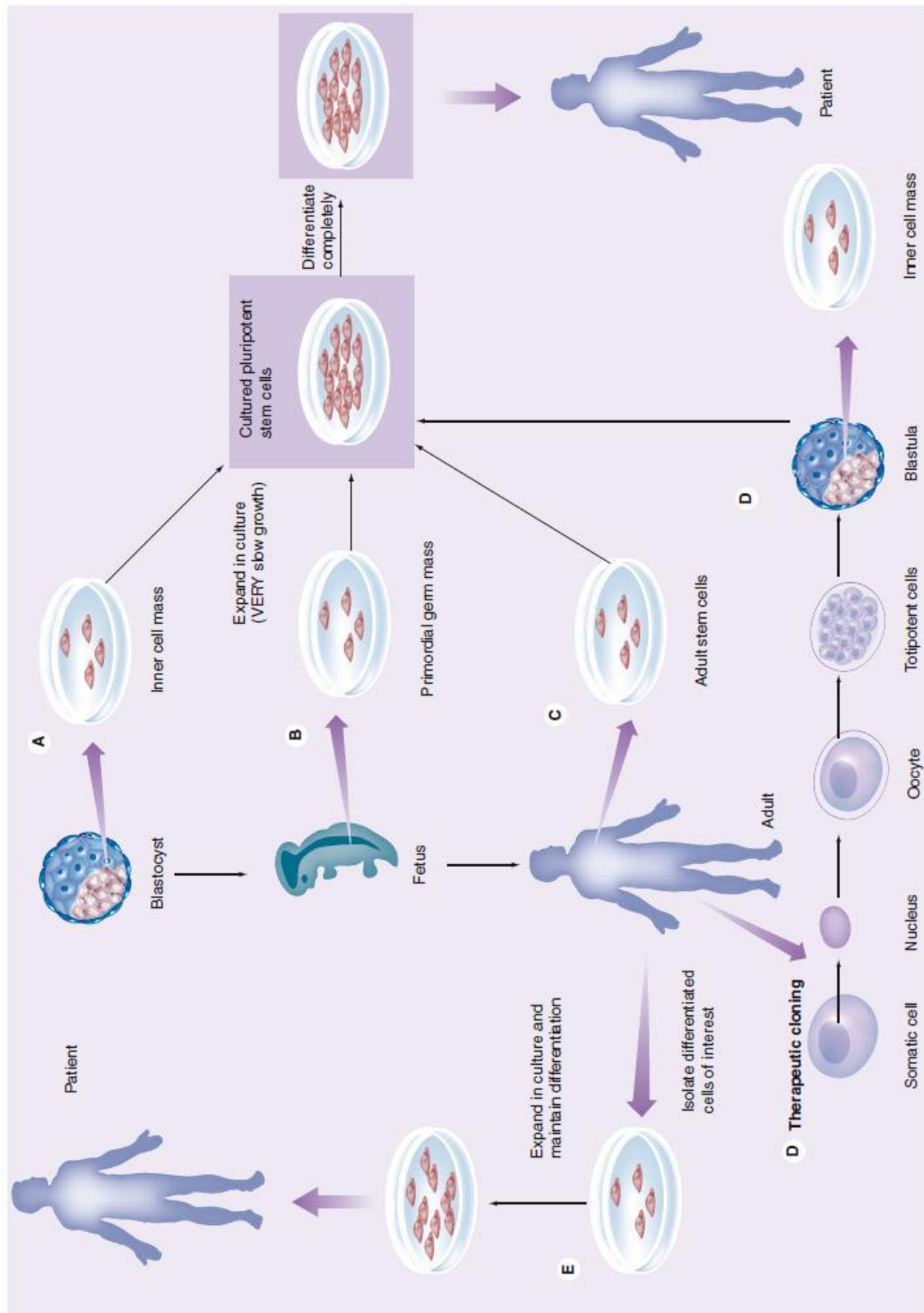
An ideal cell source should be non-immunogenic, highly proliferative, easily accessible, and should have the plasticity for differentiation into a broad range of tissue-specific cell types. Furthermore, regenerative therapies should utilize cell sources that allow for the extensive expansion of relatively few cells into large volumes of tissue [63]. Depending on the developmental stage, cells currently used in tissue engineering can be divided into four different classes: embryonic stem cells, adult mesenchymal stem cells, tissue-specific progenitor cells, and adult tissue specific cells **[Figure 15]**.

Such cells can be isolated from the host itself (autologous), from a foreign donor of the same species (allogenic), from another species (xenogeneic), or derived from an immortalized cell line. All of these cell sources have advantages and limitations for use in tissue regeneration.

Some cell types derived from adult primary human tissues, such as muscle cells, are relatively accessible, have good proliferation capacity *ex vivo*, and possess reduced immunogenic potential in the case of autologous transplantations. However, other adult cell types such as osteoblasts or cardiomyocytes are relatively limited in number. Their accessibility and *ex vivo* propagation are very difficult. Moreover, the harvesting of allogenic cells is often invasive, painful for the donor, and associated with the high risk of postoperative infection. All these logistical disadvantages and economic considerations, namely the high pre- and postoperative costs, decrease the attractiveness of allogenic cell transplantations.

Xenogeneic cells derived from primary animal tissues are almost unlimited in supply and have physiological and cytological similarity with primary human cells. For example, results of clinical trials revealed that foetal porcine cells can survive and even produce insulin when transplanted into human diabetic patients [97]. However, the risk

of the retroviral transmission and high immunogenic potential make clinical use of xenogeneic cells is controversial.



**Figure 15: Different cell sources and ways of implantation in regenerative medicine. Picture adopted from [94].**

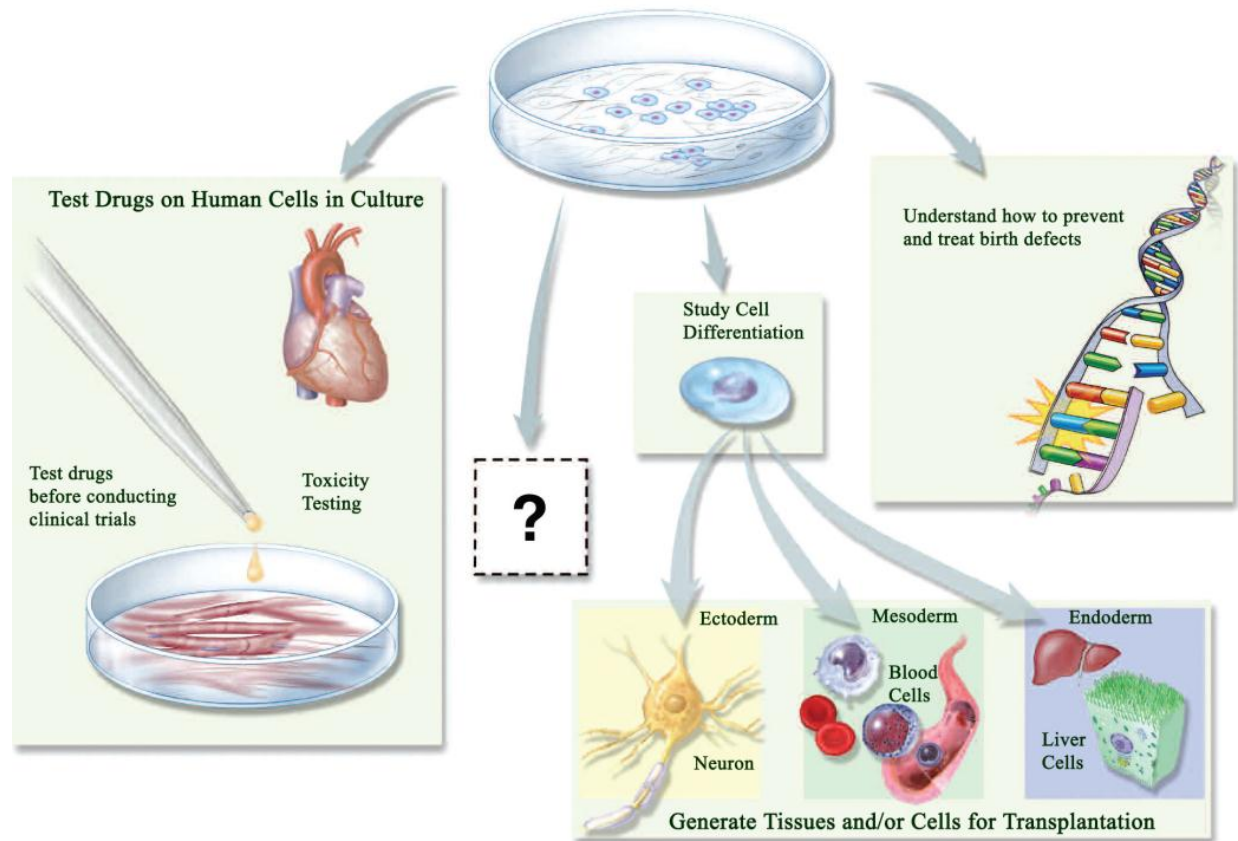
Allogenic and xenogeneic cell lines represent cells that were immortalized through incorporation of specific chemical agents or viruses [66]. Cell lines can be expanded to an almost unlimited number of cells while removing the need for further isolation. However, by indefinitely proliferating cells through uncontrolled genetic modification, daughter cells run the risk of diminished function or gaining oncogenic potential. Such risks and a lack of knowledge regarding functional limits impede the use of immortalized cell lines for *in vivo* transplantations [98]. As such, immortalized cell lines are relegated to *in vitro* modelling of tissue-like systems.

In summary, despite the impressive pre-clinical and clinical results of the last two decades, adult tissue specific cells and tissue-specific progenitor cells cannot be considered sufficient for human tissue reconstruction. Due to the invasive harvesting procedure, relatively limited quantities, and restricted proliferative capacity, clinical applications of human tissue-specific cells, even in the case of autologous transplantations, remain very controversial. Furthermore, because of the high risk of immunorejection, today's transplantation of allogenic and xenogeneic cells is followed by immuno-suppressive therapies [94].

## Stem Cells

Due to their advantageous properties of self-renewal, immunomodulation, and plasticity, stem cells are the most attractive candidates for human tissue engineering and cell therapy. There are four main fields for stem cell application in the regenerative medicine: cell therapy, *in situ* tissue engineering, *ex vivo* tissue engineering, and *in vitro* testing [Figure 16].

Depending on application, stem cells can be either transplanted directly to the site of injury as cell suspension or combined with biocompatible and biodegradable scaffolding material prior to transplantation. Stem cells also can serve as building blocks for *extracorporeal de novo* generation of complex functional tissue or organ substitutes. Additionally, organotypic cultures containing stem cells can be used as test objects for evaluation drug effects as well as for simulation of *in vivo* physiological and pathological processes *in vitro*.



**Figure 16: Strategies for application of human stem cells in regenerative medicine. Picture adopted from [13].**

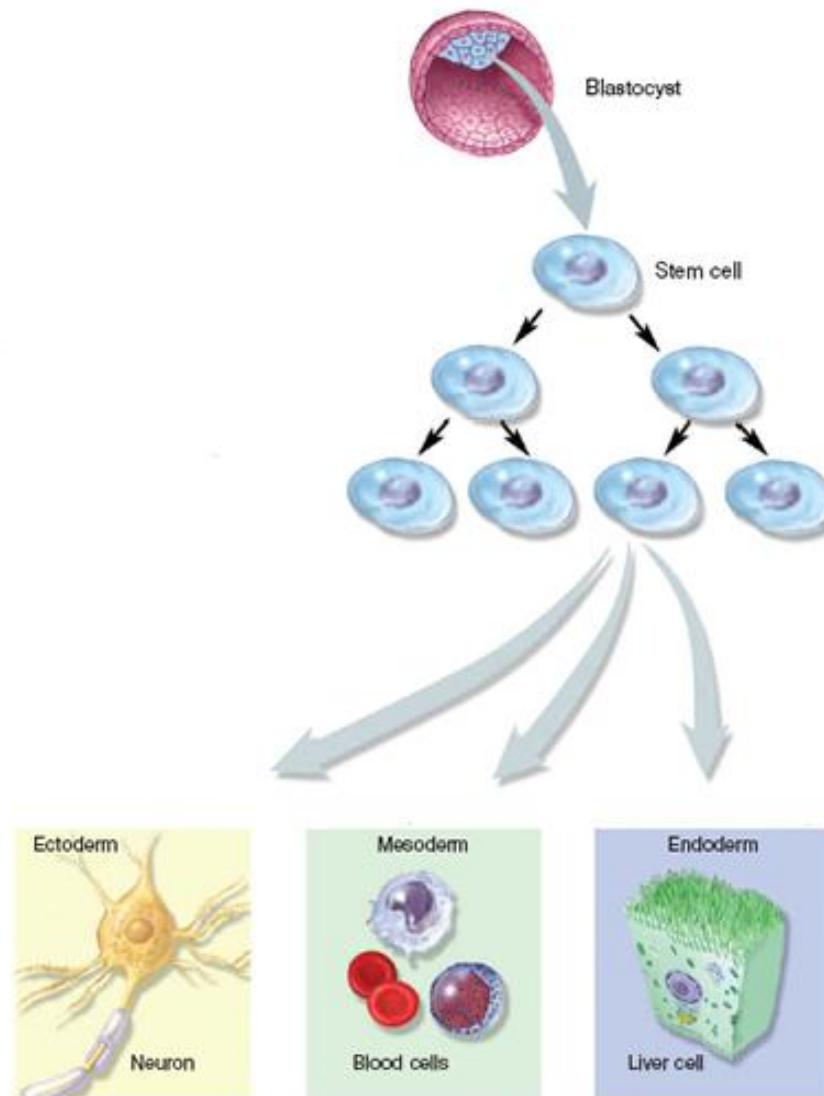
### Embryonic stem cells

Embryonic stem cells (ESCs) are a promising cell source for human tissue regeneration [99]. These cells are pluripotent and give rise to all somatic cell types in the human body. The growth potential of ESCs is almost unlimited because they can divide indefinitely [100]. Pluripotent ESCs do not freely exist in the human body; they must be isolated either from inner cell mass of the blastocyst or during termination of pregnancy from abortive foetal tissues **[Figure 17]**.

Due to their foetal origin, ESCs express low levels of major histocompatibility complex (MHC) I, consequently lacking immunogenic potential [99, 101]. However, the spontaneous differentiation of ESCs into embryoid bodies (EBs) *in vitro* leads to a 2- to 4-fold increase in MHC I expression. Prior to ESC use in clinics problems stemming



from their isolation, propagation and purification as well as their controversial immunoneutrality and tumorigenic potential need to be addressed. ESCs are also associated with prominent ethical concerns.

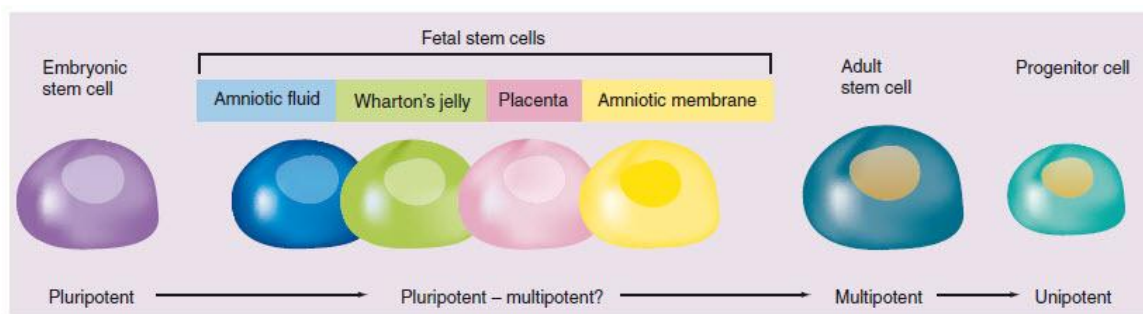


**Figure 17: Potential of embryonic stem cells for regenerative medicine. Picture adopted from [13].**

### **Human perinatal extra-embryonic mesenchymal stem cells**

Within the last decade, perinatal extra-embryonic tissue-derived stem cells were considered a viable cell source for regenerative medicine [102, 103]. These cells, of

mesenchymal phenotype, are obtained during gestation from amniotic fluid or after termination of gestation from aborted material. Additionally, human perinatal cells can be isolated immediately after delivery from extra-embryonic tissues like foetal membranes, placentas, umbilical cords, amniotic fluid, and human umbilical cord blood. As, extra-embryonic tissues are normally discarded after birth, the isolation of these cells is free of the ethical concerns that incarcerate other stem cell types [102]. Human extra-embryonic mesenchymal stem cells (EE-MSCs) can be of foetal or maternal origin. Depending on that they exhibit a slightly variable phenotypic profile [104-106]. Currently, foetal EE-MSCs are considered to be stem cells with features that combine the pluripotent properties of ESCs and multipotent properties of adult mesenchymal stromal cells (MSCs) [Figure 18].

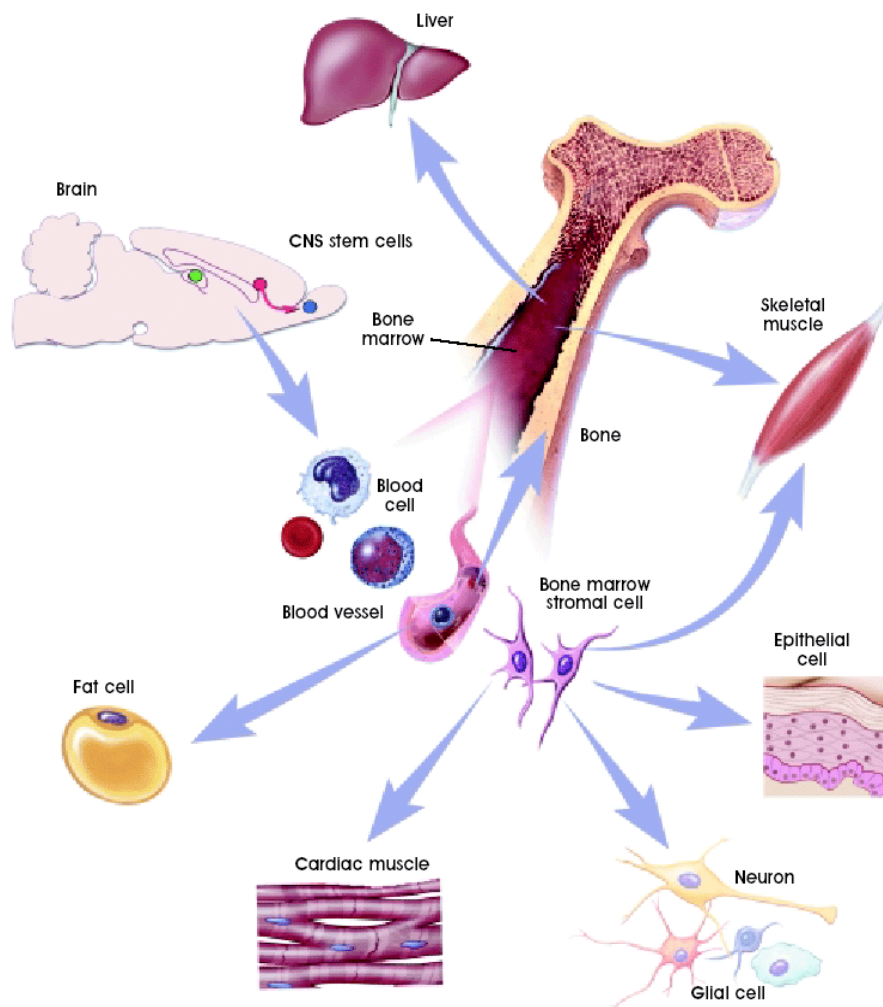


**Figure 18: Ontogenetic development stages of human perinatal mesenchymal stem cells. Picture adopted from [103].**

Due to the close ontogenic relationship of embryonic stem cells with MSCs derived from extra-embryonic tissues, amniotic fluid, and umbilical cord blood, embryonic stem cells might have immunoprivileged characteristics, a broader multipotent plasticity, and a faster proliferation than adult MSCs [103]. Furthermore, unlike totipotent ESCs, multipotent EE-MSCs do not show any sign of teratoma formation after implantation *in vivo*. However, current experimental data characterizes EE-MSCs as a particularly interesting cell type for potential clinical utility and bio-banking, as their biomedical potential is only partially elucidated.

## Human postnatal mesenchymal stem cells

Almost all human postnatal tissues contain varying amounts of multipotent MSCs that reside in niches and normally differentiate into specific cell types of a given tissue. Postnatal stem cell types are largely of a mesenchymal phenotype and can be differentiated into multiple specialized lineages, such as adipogenic, chondrogenic, osteogenic or myogenic, for instance [Figure 19].



**Figure 19: Differentiation potential of adult mesenchymal stem cells. Picture adopted from [13].**

Compared to human ESCs, human perinatal and postnatal MSCs possess a significantly reduced self-renewal and differentiation potential [107, 108]. It is challenging to characterize these cell types, as there is no unique marker that would allow their specific

prospective isolation. According to a minimal criteria for defining human MSCs, as proposed by the International Society for Cell Therapy, human MSCs should at least be adherent to the tissue culture polystyrene (TCPS) and be differentiable towards adipogenic, chondrogenic, and osteogenic lineages. Additionally, they should possess specific phenotypes characterized by the expression of clusters of differentiation like CD73, CD90, and CD105, and the lack of expression of CD45, CD34, CD14, and HLA-DR. Experimental analysis revealed that MSCs derived from different tissues exhibit slightly different surface marker expressions, indicating that these cells might have also slightly different features and capacities. Moreover, the proliferation and differentiation capacity of perinatal and postnatal MSCs can vary depending on tissue type, donor age, or foetal development stage, respectively. Contrary to previous assumptions, these cells are immunogenic. Their implantation is only possible in conjunction with immunosuppressive drug administration.

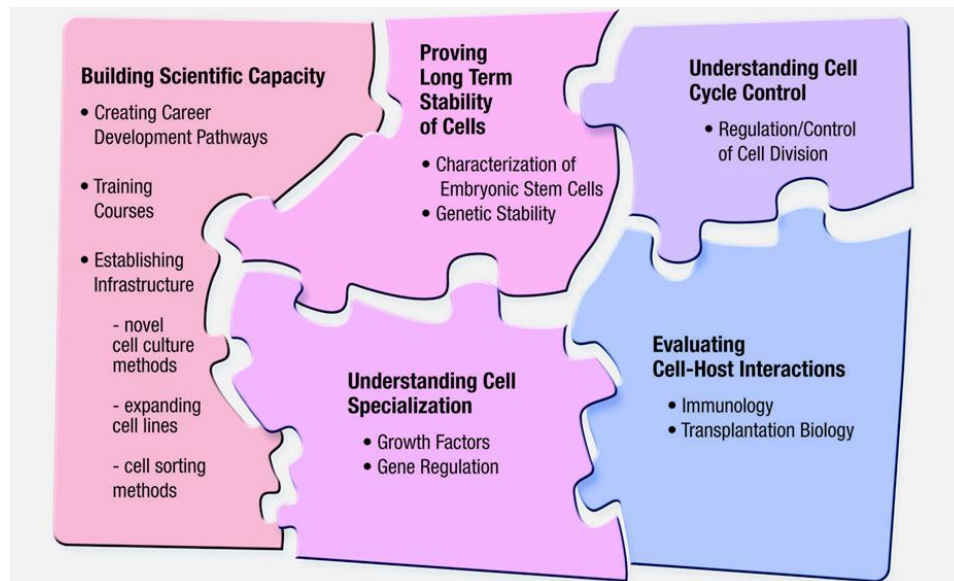
Despite these hurdles, significant progress in using human adult MSCs has been demonstrated during the past two decades. So far, bone marrow transplantation is the most successful, clinically-approved method of cell therapy. Starting from early 1990's, adult MSCs have been used in the treatment of acute and chronic graft-versus-host disease (GVHD). Moreover, bone marrow transplants containing a mix of mesenchymal and hematopoietic stem cells are described to repopulate damaged, diseased or malignant cells with fresh cells, which can later be specifically instructed to differentiate into the appropriate tissue or blood cell types. It has been shown that allogenic bone marrow MSC transplants have potential in the treatment of leukaemia, myocardial infarction, pancreatic regeneration, neurological disorders, and inherent abnormalities, such as osteogenesis imperfecta [109]. An increasing number of scientific data as well as first successful results of clinical trials demonstrate that the therapeutic effects of MSCs might not be related to their differentiation capacity, but rather, to their immunomodulatory potential and the release of paracrine signals [110, 111]. Although promising, stem cell therapies using human MSCs are not yet established as routine clinical methods. The clinical translation of MSCs is still blocked by various obstacles. Most prevalent among them are the still unclear associations between phenotypic

characteristics and biological functions of MSCs, as well as a lack of data about long-term safety, transplant actions *in vivo*, and transplant efficacy in the clinical setting.

### Challenges with the clinical use of stem cells

Stem cells, in combinations with smart biomimetic materials, have been used to establish novel cell-based therapies and tissue-engineered products.

Despite promising achievements, the critical issues hampering broad the clinical applications of such strategies need to be solved. Among them, the long term survival of transplanted stem cells in host tissue, homogeneity of stem cell populations, control of stem cell migration fate and function after transplantation, and the establishment of a treatment regimen for stem cell-replacement therapies are the most pressing of issues [Figure 20]. After developing new, clinically-relevant methods for the isolation and purification of stem cell populations, their propagation in clinically-relevant, serum- or xenogeneic-free environments and subsequent long-term freezing will be necessary in establishing functional non-immunogenic stem cells. Moreover, a better understanding of the epigenetic mechanisms participating in self-renewal and differentiation as well as in the immunogenic response of stem cells will be necessary for their effective clinical utilization.



**Figure 20: Obstacles on the way for broad clinical use of stem cells. Picture adopted from [13].**

## 1.12. BIOMATERIALS FOR TISSUE ENGINEERING

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An ideal biomaterial for *in vitro* drug testing, toxicological analysis, cell banking, and reconstruction of damaged or diseased human tissues *in vivo* should be non-immunogenic, biocompatible, highly reproducible, approvable, and affordable. Moreover, such scaffolds should replicate features of native human tissue and allow for the fast regeneration of target tissue [112]. Biocompatibility is the most critical characteristic for the successful clinical use of a concrete biomaterial. According to Williams' 1987 definition, biocompatibility is the ability of a material to perform with an appropriate host response in a specific application. A biomedical material is considered to be biocompatible if it actively resists blood clotting and bacterial colonization. Furthermore, it should not aggravate the healing process. Unfortunately, though a broad range of biomaterials are used for biomedical applications [95], many induce a non-specific, stereotyped biological reaction.

Biomaterials can be divided into five major classes: polymers, metals, ceramics (including carbons, ceramics, and glasses) as well as plant- and animal-derived materials, and human tissue-derived scaffolds. All classes of biomaterials can be characterized by six parameters: type of bulk material, dimension of architecture and porosity, physico-chemical surface composition, mechanical properties, type of initial scaffold environment, and degradability [95, 113, 114]. Due to their versatility, biomaterials can play different roles. For cell-based implants biomaterials can serve as space holders. In such situations, they prevent incorporation of surrounding tissues into the body of implant or facilitate attachment, survival, migration, and controllable differentiation of stem cells. Moreover they can serve as clusters for vascularisation, formation, and remodelling of *de novo* generated tissue as well as vehicles for delivery of cells to the damaged tissue. They can also facilitate retention and distribution of specific cells throughout the injury sites [113, 114].

### **Natural biomaterials**

Allogenic and xenogenic bone, skin, intestinal mucosa, and human foetal membranes, are tissues that have been employed for the generation of decellularized ECM. The human foetal membrane is one of the oldest scaffolds used in tissue engineering

applications. Due to its bacteriostatic, anti-angiogenic, anaesthetic, immunosuppressive properties, and its ability to inhibit scarring and promote wound healing, the human amniotic membrane can be considered as an ideal scaffold for surgery and wound healing management [115]. Currently, cryopreserved amniotic membranes (Cryo-AM) are widely used in surgical applications because of their clinical efficacy and safety [116]. Biopolymers represent the biggest class of materials used in medical applications. The development of polymeric biomaterials can be considered as an evolutionary process. Reports on the applications of natural polymers as biomaterials date back thousands of years [117]. They can be derived from natural components or manufactured from synthetic organic processes. Currently polymeric biomaterials are widely used for the production of orthopaedic, dental, soft-tissue, and cardiovascular substitutes and implants [95].

Natural biopolymers are very similar or even identical with macromolecular substances of natural biological environment. Most of today's natural biopolymers are constituents of the ECM obtained from different animal tissues, such as tendons, muscles, cartilages, and skin. These biopolymers include collagen, glycosaminoglycans, gelatine, fibrin, heparin, and hyaluronic acid. Furthermore, natural biopolymers can be manufactured from such plant components as cellulose, sodium alginate, and natural rubber. Due to their inherent biological properties, natural biopolymers are non-toxic and do not provoke chronic inflammatory reaction. Furthermore, because of the similarity to naturally occurring components natural biopolymers are able to function on the molecular level. The greatest disadvantages of biological polymer scaffolds are batch-to-batch variations and limited versatility for designing of exogenous ECM with specific well defined properties, immunogenicity, spontaneous degradation by natural enzymes, and pyrolytic decomposition by temperatures below the melting point [95].

### **Synthetic biomaterials**

Modern synthetic biopolymers range from hydrophobic, non-water-adsorbing materials, such as silicone rubber (SR) or polyethylene (PE), to hydrophilic materials, such as poly(vinyl chloride) (PVC) or co-poly(lactic-glycolic acid) (PLGA). It can be water-swelling materials, such as poly(hydroxyethyl methacrylate) (PHEMA) or water

soluble materials, such as poly(ethylene glycol) (PEG) [95]. The application of such synthetic polymers to medicine is a recent phenomenon. The use of polymeric biomaterials as we know them today started in the 1940s during the second world war [118]. During the latter half of the twentieth century, material scientists attempted to engineer novel polymeric materials and modify existing polymers that could exhibit biocompatibility and adequate mechanical properties suitable for specific biomedical applications. In addition, recent advances in biotechnology and pharmaceutical science opened novel frontiers in biomedical fields, demanding materials with bioactivity, biocompatibility, and in many cases transient existence [119]. Such polymers can be produced in large quantities and with highly reproducible properties. The *in vivo* stability of biopolymers can be tuned by modulating their chemical composition. The greatest disadvantage associated with synthetic biopolymers is their inability to specifically interact with the biological system. However, the use of biologically inert materials (materials that neither present biological signals nor respond to proteolytic remodelling) served as starting point for the engineering novel materials. Such synthetic materials can be designed to overcome the limitations associated with naturally derived materials. In recent studies, materials with specific mechanical, chemical and biological properties have been synthesized. Now, fully-defined materials are available that can be used to replace naturally derived materials for the formation of tissue like constructs *in vitro*. Their usefulness has also been demonstrated *in vivo*. Today, these materials are mainly used to study cell-matrix interactions and the presentation of biologically active molecules for therapeutic approaches. More research is required to make such materials true alternatives for naturally-derived materials, which allow cellular remodelling and tissue regeneration [79, 120]. Nonetheless, synthetic biomaterials hold great promise in becoming the basis for the *de novo* generation of engineered tissues under well-defined conditions.

### 1.13. NEW STRATEGIES FOR TISSUE ENGINEERING

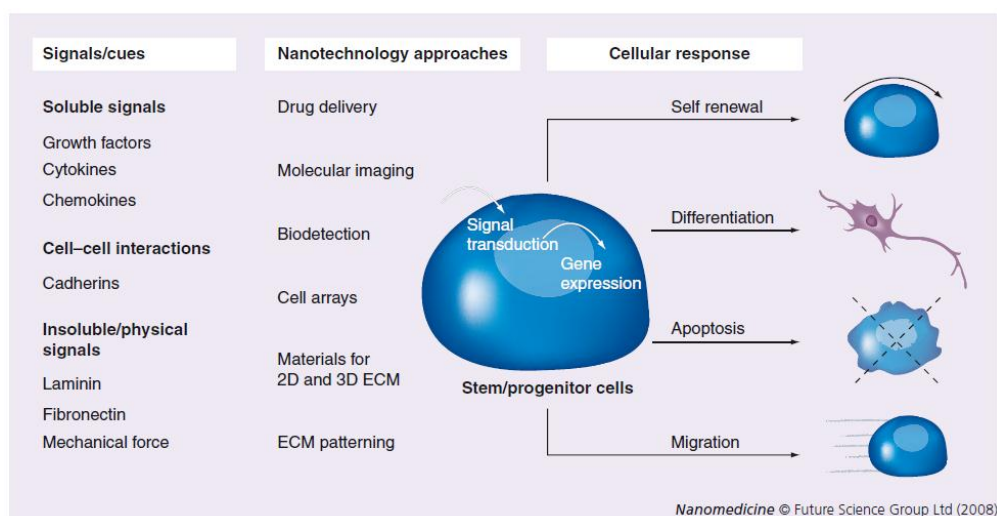
Novel tissue engineering strategies developed over the last decade include the use of smart stimuli-responsive materials such as intelligent hydrogels and polymers. Recent experimental results have shown that though such approaches are very profitable,



several critical conditions must be still considered namely, stimuli-responsive materials must interact with proteins and cells without interfering with their biological activities, maintain their features after implantation, and be non-toxic and non-tumorigenic to surrounding cells and the microenvironment [121].

### Stimuli - responsive, “smart” materials

From the tissue-engineering point of view, stimuli-responsive materials allow for the generation of 3D matrices, such as hydrogels and 2D coatings, assembled from polyelectrolyte multilayers, with the potential to control spatial organization, adhesion, migration, functionality, and integration of cells [Figure 21].



**Figure 21: Possible application of stimuli-responsive materials in regenerative medicine. Picture adopted from [121].**

Major advantage of stimuli-responsive materials, compared to conventional biomaterial scaffolds, is their unique ability to regulate the spatial-temporal interactions at the cellular level [121].

“Smart” materials might respond to specific stimuli, such as temperature, pH, light, electric field, magnetic field, and so forth. Dependent on the physico-chemical composition of the material and kind of the stimulus, “smart” scaffolds can dramatically change in terms of shape, structure, solubility, adhesiveness, and transition, to name a few [122]. For example, “intelligent hydrogels” can exhibit swelling depending on

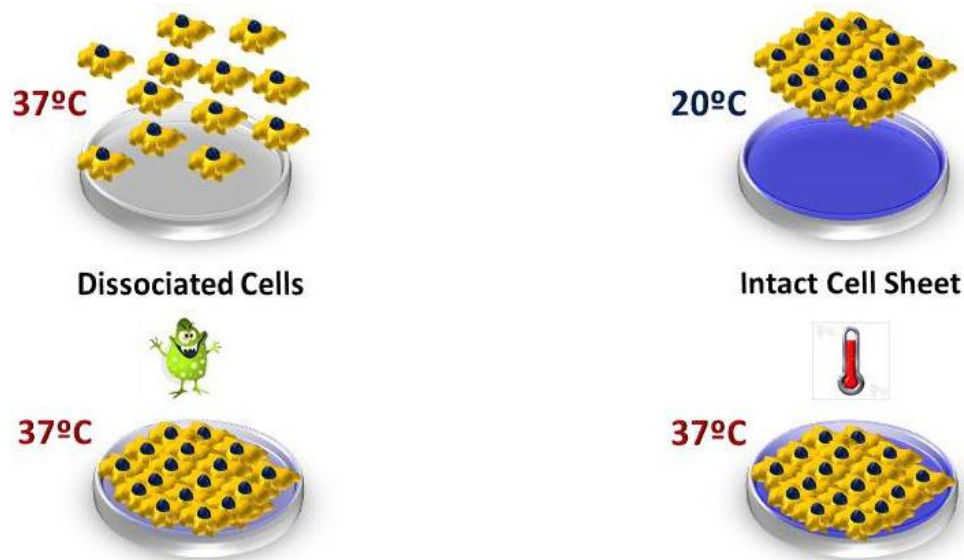
external environmental conditions. Moreover, the response of “smart” scaffolds on a specific stimulus can be tuned by changing of the micro-environmental properties. Current applications of stimuli-responsive “smart” materials include the engineering of tuneable scaffolds for controlled adhesion, propagation, spatial organization, or differentiation of cells, scaffolds for regulation of the bimolecular environment, functional self-assembled scaffolds, scaffolds for controlled and targeted drug delivery, and scaffolds enhancing tissue regeneration. A widely used example of such “smart” polymer is hydrogels, which collapse in water after the decrease of environmental temperature. Such thermo-responsive poly-N-isopropylacrylamide (PNIPAAm) coatings are also used for cell sheet engineering [95].

### Cell sheet engineering

Cell sheet engineering is a new, versatile, and flexible scaffold-free method for the delivery of cells. This method is based on enzyme-free harvesting of contiguous viable cell monolayers from “smart” biomimetic coatings through the application of specific stimuli. Single cell sheets can either be applied directly or after assembly into three-dimensional tissue-like structures consisting of multiple cell layers. By the choice of the cell source and the arrangement of cell sheet structures, heterotypical cell assemblies can mimic the *in vivo* arrangement. Currently, three different platforms for the detachment of contiguous cell sheet are applicable: temperature, magnetic field, and electrical charge [80]. The most prevalent approach among these three is cell sheet engineering on thermo-responsive poly-(N-isopropylacrylamide) (PNIPAAm) grafted culture dishes [**Figure 22**].

Under normal culture conditions, the surface of such dishes is relative hydrophobic and cells attach, spread, and proliferate similarly to the normal tissue culture polystyrene. However, once the environmental temperature is reduced below the polymer’s lower critical solution temperature (LCST), the surface will be converted to a hydrophilic one. This leads to the swelling of the coating and the formation of a hydrated layer between the dish and the attached cells [123]. This effect allows spontaneous detachment of cell sheets without the need for an enzymatic treatment, such as trypsinization or accutesation.

Cell sheet engineering that utilized thermo-sensitive PNIPAAm surfaces was introduced by Teruo Okano over a decade ago and has been successfully pre-clinically applied in the reconstruction of ocular surfaces, periodontal ligaments, oesophagus, cardiac muscle, bladders, and bones, as well as for treatment of diabetes mellitus [124-126]. Moreover, the cell sheets peeled from thermo-responsive polymer surfaces show promise for polysurgery, a method of repetitive transplantation for up to 30 multilayered cell-sheet grafts, allowing, for example, the generation of 1 mm-thick myocardial tissue-like structures [127].

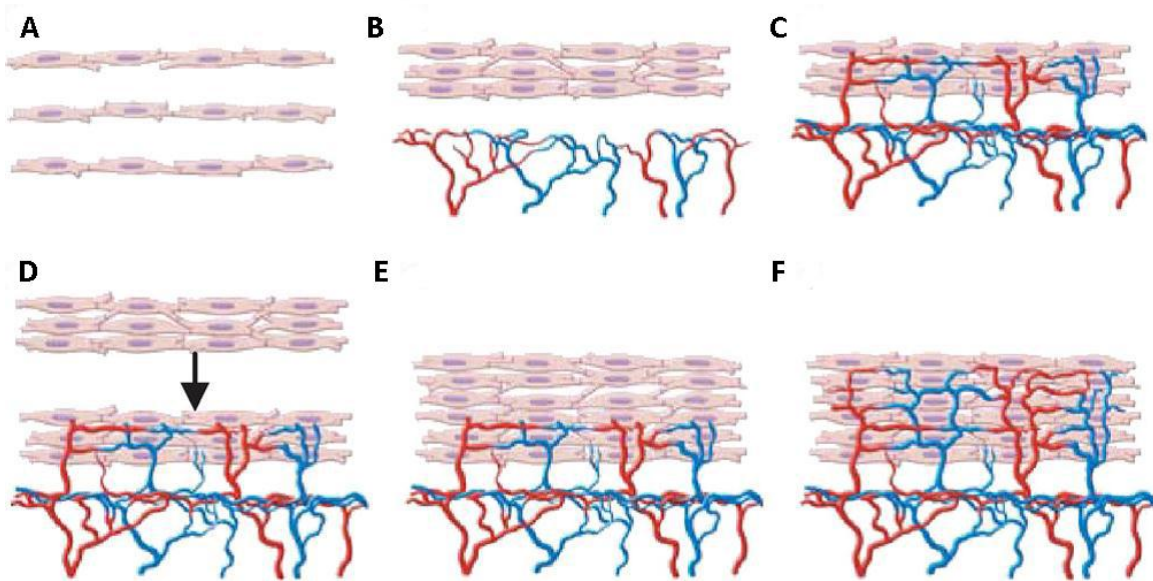


**Figure 22: Cell sheet engineering on thermo-sensitive biomimetic surfaces. With typical proteolytic harvest by trypsinization, both ECM and cell-to-cell junction proteins are degraded for cell recovery (A). In contrast, cells harvested from temperature responsive dishes are recovered as intact sheets along with their deposited ECM, by simple temperature reduction (B). Picture modified from [123].**

Transplantation of multi-layered “polysurgical” structures over arteries or veins has been shown to induce the formation of a vascular network that could be ectopically connected to the host vasculature [Figure 23].

Additionally, currently performed studies demonstrated that 3D tissue-like constructs composed from stacked cell sheets can be pre-vascularized *in vitro* by incorporation of sparsely cultured human umbilical vein endothelial cells (HUVEC) [128, 129]. It has

been reported by Sasagawa et al. that after sandwiching of between two sheets of myoblastic cells inserted HUVEC started to sprout and form network structures [129]. Moreover, endothelial networks incorporated into the multiple structures composed from five sheets of myoblasts were observed to connect to the host vasculature after subcutaneous transplantation into the nude rats. In the second study performed by Asakawa et al. single mono-cultured EC sheets were sandwiched between two layers of normal human dermal fibroblasts (NHDF) in different orders. Pre-vascular networks formed by HUVEC were observed in all such triple-layer constructs [128].



**Figure 23: Principle of polysurgery approach. (a) Individual cardiomyocyte sheets are harvested from temperature-responsive culture dishes and stacked to create layered constructs. (b) Triple-layer myocardial grafts are transplanted subcutaneously. (c) After transplantation, neovascularization occurs within the bioengineered tissues. (d) After sufficient neovascularization has occurred, a second triple-layer myocardial graft is transplanted directly over the first construct. (e) Neovascularization of the second graft occurs through the first construct, creating thick tissues that can overcome the limits of passive diffusion. Picture adopted from [127].**

### Cell sheet engineering on polyelectrolyte multilayer coatings

Cell sheet engineering on polyelectrolyte multilayer coatings, i.e. PEM-based cell sheet engineering, presents an alternative approach to the aforementioned issues.

Polyelectrolyte multilayer hydrogels (PEM), first introduced in 1992, represent a specific type of “smart” 2D coating generated by self-assembly. PEM self-assembly is a repetitive, alternating, layer-by-layer adsorption of cationic and anionic polyelectrolytes from dilute aqueous solutions. Their deposition is a significant and versatile means of modifying biomaterial surfaces [130].

The preparation of PEMs is simple and widely applicable. The electrostatic interaction between the oppositely charged polyelectrolytes drives the build-up of multilayers, which occurs upon dipping material into polymer solution or spraying of polymer solution on the material. The PEM modification can be done with materials of any size, shape, or texture. The method allows the build-up of coatings from several nanometre to even micrometre thickness that exhibit a long-range order and stability, whereas the layers are highly interpenetrated at short range [131, 132]. Generally, PEM coatings can be engineered for specific bio-affinities and deposited in a high spatial order [133]. Applications of PEMs currently include bio-sensing in microarrays, cell encapsulation, and serving as carrier systems for bioactive agent delivery.

Classical strategies have assured continuous progress in tissue engineering during the last two decades, shown necessary relevance for clinical applications, and been used successfully in patient care in some instances [64]. However, routine clinical applications of tissue engineering products are hampered by the absence of preformed vascularization, immunological rejection, high risk of infections, and malignancy [113, 134]. These factors play a critical role in tissue integration, cell necrosis, and implant rejection. Additionally, the “scale-up” of classical tissue engineering strategies might be limited by restricted availability and high costs of biological materials [135]. Failure of engineered tissue substitutes are induced by immunological rejection, cell necrosis, inflammation or associated postoperative infections and can lead to chronic inflammation, invalidity, or even death, and thus reduces the potential of the tissue engineering approach [96, 136]. For this reason, novel strategies, fashioned during last decade, aim to generate tissue substitutes in the absence of biomaterials. Scaffold-free tissue engineering and tissue engineering using stimuli-responsive scaffolds are prominent examples of such efforts. Scaffold-free constructs consist of different cell types that, by self-arrangement, form tissue-like assemblies. The controlled patterning

of cells and the formation of vascular structures inside cell masses, which go much beyond the limits of oxygen diffusion and the transport of nutrition and waste products, would be a critical step towards the formation of more functionally-engineered tissues and their efficient connection to host vascularisation systems.

## CHAPTER 2

# SCOPE

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Correct development, as well as following engraftment and survival of complex tissue-like substitutes generated *de novo*, are among the most critical aspects of modern regenerative medicine and perhaps the most ambitious aim of tissue engineering.

In the context of my thesis a polyelectrolyte multilayer (PEM) based methodology is developed for engineering and harvesting of human extra-embryonic perinatal mesenchymal stem cell sheets. This new technology provides an alternative for the platform based on thermo-responsive polymers. The combination of the unique regenerative potential of perinatal MSCs with advantageous properties of cell sheet engineering will allow designing of biocompatible, cost effective tissue-like substitutes for regenerative medicine. The basis of my thesis is a new method that allows for the harvesting of intact cell sheets by electrochemical means. In combination with micro-patterning techniques this method also allows for the control of the spatial organization of cells in two dimensions.

First results section (Chapter 4) describes the protocols and critical parameters for routine and effective isolation, long-term cultivation, and maintenance of human term placenta-derived mesenchymal stem cells (PD-MSCs). In order to evaluate their regenerative potential, isolated human PD-MSCs were systematically characterized in terms of: phenotype, genotype, growth and mortality rates, as well as mesodermal plasticity.

The next results section (Chapter 5) contains the design of the functional polyelectrolyte multilayer (PEM) based platform for assembling of cell sheets from human PD-MSCs and the systematic characterization of the resulting PD-MSC sheets in terms of their regenerative potential.

The final results section (Chapter 6) is summarising optimal conditions for the non-invasive peeling of intact PD-MSC sheets by electrochemical means. In addition, this chapter also contains the characterization of the electrochemically peeled PD-MSC sheets in terms of: viability, adhesion capacity, and mesodermal plasticity. As the evaluation of the viability, the in-growth, and the regenerative potentials of PD-MSC sheets after implantation is also very important for the final application this chapter finishes with experiments dealing with the instructed differentiation by co-culture with adult human osteoblasts. Such co-cultures can simulate the effect of stem cell sheet –



host tissue contact *ex vivo* and thus clarify whether the PD-MSC sheets indeed have potential for tissue regeneration.

Overall I believe that the results of my thesis lead towards a principally new, very effective and practical method for the creation of three-dimensionally designed complex tissues for transplantation surgery, drug screening procedures and cell based therapy.

## CHAPTER 3

# MATERIALS AND METHODS

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### 3.1. ISOLATION OF HUMAN PLACENTAL MSCs

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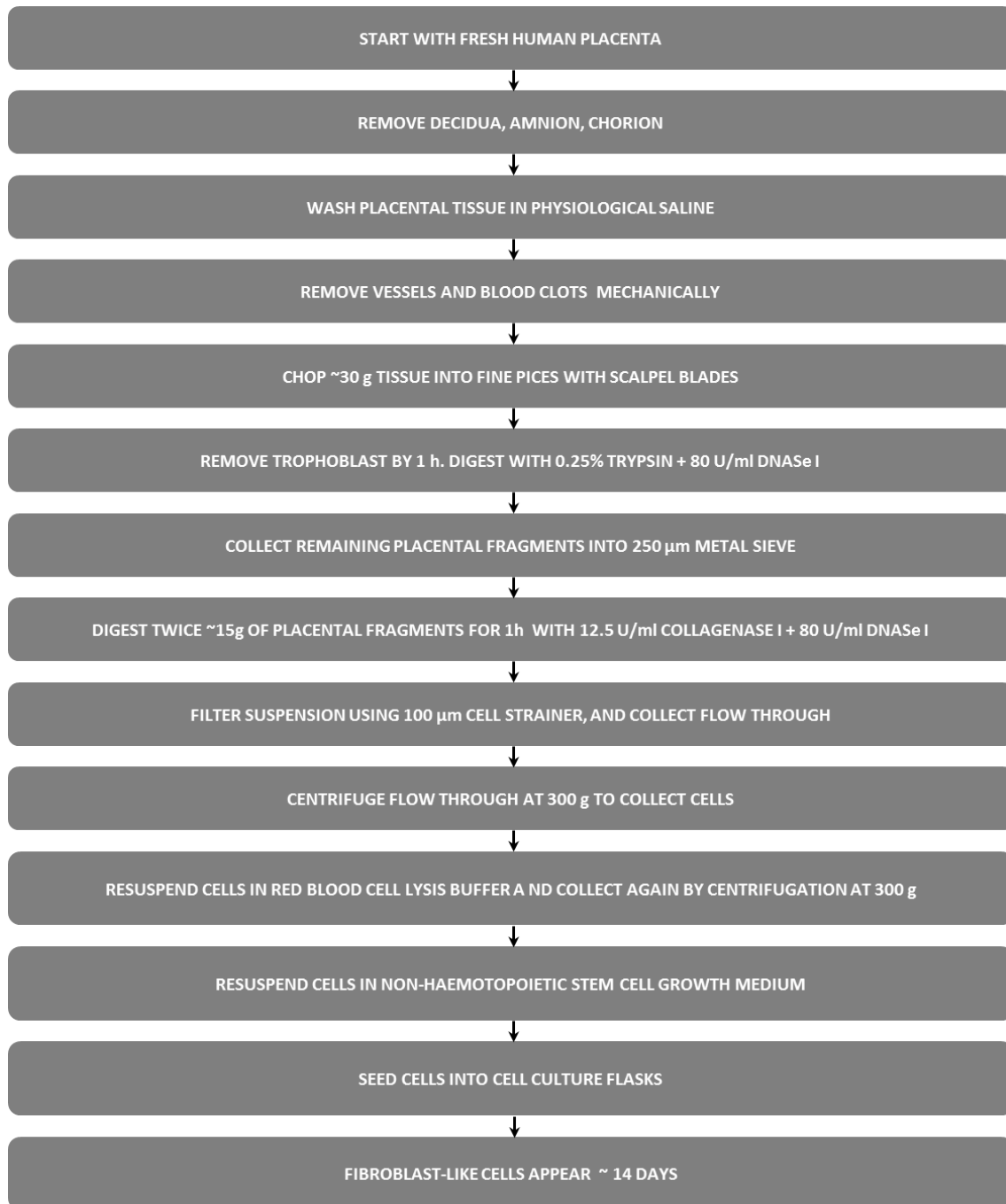
*In the first part of the chapter I am describing the materials and methods, which have been used for the isolation, characterization, and long-term cultivation of placenta-derived mesenchymal stem cells (PD-MSCs).*

#### 3.1.1. Placenta collection

The Ethical Committee of the District of Zurich approved the protocol (study Stv22/2006). Following written consent, placentas, umbilical cords, uterus muscle biopsies, and umbilical cord blood samples were collected immediately after elective caesarean section in the absence of labour, preterm rupture of membranes, chorioamnionitis, or chromosomal abnormalities. Mean maternal age was 32 years (between 28 and 39 years) and mean gestational age was  $38 \pm 1$  weeks. Mean placental weight was  $573 \pm 113$  g.

Isolation procedure is depicted on the **[Figure 24]**. After removal of decidua and foetal membranes, approximately 30g placental tissue was minced and washed three times in physiological saline. Blood vessels and clots were removed mechanically. The minced placental tissue was subjected to sequential digests with trypsin and collagenase I. First, to remove the trophoblastic epithelial cell layer, tissue was incubated in 50mL 0.25% trypsin solution containing 80U/mL DNase I (Roche AG, Basel, Switzerland) for 1 h at 37°C. The remaining placental fragments were separated in a 250µm metal sieve from the 'trypsin cell suspension'. Approximately 15g of placental fragments were subjected to a second digest with collagenase. For that, tissue fragments were incubated with 50mL of 12.5U/mL collagenase I (Sigma-Aldrich AG, Buchs, Switzerland), 80U/mL DNase I for one hour at 37°C. Cell suspensions from both trypsin and collagen digests were filtered twice through 100µm cell strainers (BD Bioscience, San Jose, CA), then the cells were collected by centrifugation for 5min at 300 g. The cell pellets were shortly re-suspended in hypotonic red blood cell lysis buffer (physiological saline with 2mM EDTA, 0.5% BSA, without Ca und Mg, diluted 1:10 with distilled water), pelleted again by 5min centrifugation at 300g. Finally, the cells were suspended in 10ml Non Hematopoietic

Stem Cell Expansion Medium (NH expansion medium; Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) and plated into a single 75cm<sup>2</sup> tissue culture flask (TPP AG, Trasadingen, Switzerland) and cultured at 37°C.



**Figure 24: Fractionation scheme for isolation of MSCs from term human placental tissue**

### 3.1.2. Propagation of PD-MSCs *in vitro*

#### Colony assay

Freshly isolated PD-MSCs, passage 0, were plated at low density, i.e. 50 cells per well of 6-well plates (TPP), and cultured at 37°C, 5% CO<sub>2</sub> in Non-Hematopoietic Stem Cell Expansion Medium (NH expansion medium). Outgrowing colonies of spread cells were visualized and counted by fluorescence microscopy using rhodamine-labelled phalloidin to stain actin cytoskeleton (Invitrogen, Basel, Switzerland), and 4'6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR) to stain cell nuclei. Images were acquired with a Zeiss Axiovert 200M (Carl Zeiss AG, Feldbach, Switzerland) equipped with a digital camera AxioCam MRc, (Carl Zeiss AG, Feldbach, Switzerland).

#### Growth kinetics

PD-MSCs of passages 1, 10, and 20 taken from four different cases were plated at  $5 \times 10^3$  cells per well in 12 well-plates (TPP) and cultured at 37°C and 5% CO<sub>2</sub> in Non-Hematopoietic Stem Cell Expansion Medium (NH expansion medium). All experiments were performed in triplicate. Cell counts were determined after 24, 48, and 72h culture. For that, cells were detached with 0.25% trypsin solution (GIBCO-Invitrogen AG, Basel, Switzerland) and counted with a Coulter Z1 cell counter (Instrumenten Gesellschaft AG, Zurich, Switzerland). Dead cells were identified by staining with 0.4% trypan blue staining solution (Sigma - Aldrich AG).

### 3.1.3. Phenotypical analysis

#### Flowcytometry

Cells were collected by trypsinization. Reaction was performed on live cells, using  $10^5$  cells per antibody reaction. Cells were incubated for 25 min with specific antibodies at 4°C. Unstained, non-incubated cells serve as controls. Cells were fixed in 4% buffered formalin and analysed with a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). A minimum of  $10^4$  gated events, were acquired for each sample. For staining of the intracellular markers CK-7,  $\alpha$ -SMA, and vWF, cells were fixed in 4% buffered

formalin, shortly permeabilized by 10 min incubation in 0.9% saponin solution, and then incubated with specific antibodies for 25 min at 4°C.

Information about primary and secondary antibodies used for flow cytometry and immunochemistry is provided separately [Table 1].

ANTIGEN	CLONE	CONJUGATE	HOST	COMPANY
CD11b	ICRF44	PE	Mouse	Biologened
CD14	H5E2	-	Mouse	Biologened
CD19	HIB19	PE	Mouse	Biologened
CD34	AC136	FITC	Mouse	Miltenyi Biotec GmbH
CD44	G44-26	FITC	Mouse	BD Pharmingen
CD45	5B1.	FITC	Mouse	Miltenyi Biotec GmbH
CD54	HA58	PE	Mouse	BD Pharmingen
CD73	AD2	PE	Mouse	BD Pharmingen
CD79a	HM47	PE	Mouse	Biologened
CD90*	5E10	-	Mouse	BD Pharmingen
CD105*	266	-	Mouse	BD Pharmingen
CD117	YB5.B8	PE	Mouse	BD Pharmingen
CD133/1	AC133	APC	Mouse	Miltenyi Biotec GmbH
CD163	GHI/61	PE	Mouse	BD Pharmingen
CD166	3A6	PE	Mouse	BD Pharmingen
CD271	ME20.4-1H4	PE	Mouse	Miltenyi Biotec GmbH
HLA-ABC	G46-2.6	FITC	Mouse	BD Pharmingen
HLA-DR	G46-6	FITC	Mouse	BD Pharmingen
Cytokeratin-7	CAM 5.2	FITC	Mouse	BD Pharmingen
Cytokeratin-18	CY-90	FITC	Mouse	Sigma-Aldrich AG
Alpha Smooth Muscle Actin	1A4	PE	Mouse	R&D Systems
Von Willebrandt Factor	Sheep Polyclon	FITC	Sheep	Abcam plc
KDR / VEGF receptor 2	89106	PE	Mouse	R&D Systems
PIAP	H17E2	-	Mouse	AbD Serotec
Hepatocyte specific antigen	OCH1E5	-	Mouse	Abcam plc
SSEA-3	MC-631	-	Rat	R&D Systems
SSEA-4	MC813-70	PE	Mouse	R&D Systems
Oct-3/4	240408	-	Rat	R&D Systems
Vimentin	V9	PE	Mouse	Sigma-Aldrich AG
Nestin	10C2	-	Mouse	Abcam plc
E-Cadherin	36	PE	Mouse	BD Pharmingen
ESCM Tra-1-60*	Tra-1-60	-	Mouse	Abcam plc
ESCM Tra-1-81*	Tra-1-60	-	Mouse	Abcam plc
ZO-1	ZO-1-1A12	FITC	Mouse	Zymed Laboratories

**Table 1: Antibodies used for flow cytometry and immunocytochemistry**

## Immunocytochemistry

Briefly, cells on 15 mm glass cover slips (Menzel GmbH + Co KG, Germany) were fixed for 5 min in 100% acetone, then rehydrated in PBS and blocked with Ultra V Blocking buffer (Bio Vision Corporation, England). For staining for intracellular proteins, cells

were permeabilized with 0.1% Triton X-100 for 3min, then incubated with primary antibodies for 1h at room temperature. In case of using unconjugated primary antibodies, samples were subsequently incubated with secondary polyclonal FITC conjugated goat anti-mouse Ig (BD Pharmingen, Switzerland) or FITC-conjugated rabbit anti-rat Ig antibodies (Dako Cytomation, Glostrup, Denmark) for 30min at room temperature. All cell samples were additionally counterstained with DAPI. Images were taken with a Zeiss Axiovert 200M (Carl Zeiss AG) equipped with a digital camera AxioCam MRc, (Carl Zeiss AG) and analysed with AxioVs40 V 4.5.0.0 imaging software (Carl Zeiss AG).

### 3.1.4. Cytogenetic analysis

#### **Chromosome analysis**

Chromosome analysis of PD-MSCs cultures, passage 3, from six donors was performed. Briefly, metaphase chromosome preparations were performed according to standard procedures at a 400-500 GTG band level. Analysis and identification of the chromosomes was performed using a Zeiss Axioplan microscope (Carl Zeiss AG, Feldbach, Switzerland). Images were recorded with a Photometrics CCD KAF1400 camera (Photometrics, Tucson, AZ) and controlled with smart capture imaging software (Vysis, Inc., Downers Grove, IL).

#### **FISH analysis for detection of sex determination region**

Fluorescence *in situ* hybridisation (FISH) studies for detection of sex determination region (SRY) were performed using chromosome X (Xp11.1-q11.1, locus DXZ1) and chromosome Y (Yp11.1-q11.1, locus DYZ3, alpha satellite) probes Vysis (Abbott Molecular Inc., IL) according to the manufacturer's instructions (Abbot Molecular Inc., Des Plaines, IL 60018). Analysis of at least 200 metaphases and interphases was performed on PD-MSCs isolated from three male deliveries at passage 3 using a Zeiss Axioplan epifluorescence microscope (Carl Zeiss AG). Images were recorded by Photometrics CCD KAF1400 camera (Photometrics) and controlled with smart capture imaging software (Vysis, Inc.)

### 3.1.5. Multipotent differentiation assay

Cultures of PD-MSCs at passage 6 taken from three random donors were tested for differentiation towards mesodermal lineage *in vitro*. Differentiation was induced by their culture in commercial differentiation media: AdipoDiff, ChondroDiff, and OsteoDiff Induction Media (Miltenyi Biotec GmbH) following the manufacturer's protocol. Control cultures were grown in NH expansion medium (Miltenyi Biotec GmbH). After differentiation, cells were examined microscopically with a Zeiss Axiovert 200M (Carl Zeiss AG).

#### **Adipogenic differentiation**

For adipogenic differentiation, PD-MSCs were cultured at  $50 \times 10^3$  cells per well of 12 well-plates in AdipoDiff induction medium (Miltenyi Biotec GmbH) for 3 weeks, with fresh media added every 48h. Oil Red O was utilized to visualize fat droplets. Briefly, cells were washed twice with PBS, fixed in pre-cooled methanol for 5 min, rinsed with deionised water, and then incubated with 0.5% Oil Red O (Sigma-Aldrich AG) in isopropanol for 20 min at room temperature. Finally, stained cells were washed with water and analysed microscopically.

#### **Chondrogenic differentiation**

Chondrogenic differentiation was performed under micromass conditions in ChondroDiff induction medium (Miltenyi Biotec GmbH), according to the manufacturer's instructions. Briefly,  $5 \times 10^6$  MSCs were pelleted by 5 min centrifugation at 300g in a cell culture centrifuge. The micromass was kept in ChondroDiff medium for 24 days, with fresh media added every 48 h. The pellets were fixed in 10% formalin, and then finally embedded in paraffin. Staining for proteoglycans was performed on deparaffinised 5  $\mu$ m sections by 30 min incubation with 3% Alcian Blue (Sigma-Aldrich AG) in 3% acetic acid, pH 2.5. Counter stain was performed with Nuclear Fast Red (DAKO Cytomation).



### **Osteogenic differentiation**

For osteogenic differentiation,  $3 \times 10^4$  PD-MSCs were seeded in 12 well plates (TPP) and maintained in OsteoDiff induction medium (Miltenyi Biotec GmbH) for 3 weeks, with fresh medium added every 48h. After 21 days, cells were fixed in 70% ethanol. Extracellular calcium deposition was stained by 45 min incubation with 2% Alizarin Red S (Sigma-Aldrich AG) in water. Finally, stained cells were washed with deionised water and analysed microscopically.

### **Angiogenic differentiation**

Angiogenic differentiation was performed as described [137]. Briefly, PD-MSCs were seeded at the density of  $2.5 \times 10^4$  cells per well of 12 well plates and cultured for 7 days in Dulbecco's Modified Eagle Medium (Invitrogen AG) supplemented with 2% foetal bovine serum (Invitrogen AG) and 50 ng/ml vascular endothelial growth factor 121 (29). Control cultures were performed without VEGF. After 7 days, cells were fixed and stained with mouse anti-human CD34 antibodies (Miltenyi Biotec GmbH).

### **Statistical analysis**

Data are shown as mean  $\pm$  SD. Mann-Whitney (nonparametric) test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Data are given as mean  $\pm$  standard deviation. Significance level was set at  $p < 0.05$ .

## 3.2. DESIGNING OF POLYELECTROLYTE MULTILAYER SUBSTRATES

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*In the second part of the chapter I am describing materials and methods which were used for the fabrication, mechanical characterisation, and tuning of the polyelectrolyte multilayer coatings (PEM)*

### 3.2.1. Preparation of polyelectrolyte multilayer substrates

#### **Substrate cleaning**

Pre-treatment of silicate glass coverslips (for cell culture experiments) or silicate waveguides slides (for optical measurements) was done as previously described [138].

#### **Polyelectrolyte and fibronectin solutions**

Solutions of poly(L-lysine) hydro-bromide (PLL, Mw 15-30 kDa) (Sigma, AG, Switzerland) and hyaluronic acid sodium salt from bovine vitreous humour (HA, Mw 300 kDa) (Sigma, AG, Switzerland) were prepared at 1mg/ml in HEPES-2 buffer (10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.4, 0.15M sodium chloride) [139]. Solutions of poly(allylamine hydrochloride) (PAH, Mw 70 kDa) (Sigma, AG, Switzerland) and poly(styrene sulfonate) (PSS, Mw 70 kDa) (Sigma, AG, Switzerland) were prepared at 0.1mg/ml in HEPES-2 buffer at pH 7.4 [140]. Human recombinant fibronectin (FN) (Biomedical Technologies Inc., USA), was used at 50 µg/ml in HEPES-2 buffer. All solutions were sterile filtered through 0.22 µm filters before use. Fluorescent labelling of FN was performed with Alexa Fluor® 488 protein labelling kit (Invitrogen, Switzerland) following the manufacturer's protocol.

#### **Preparation of native [PLL/HA] multilayer substrates**

Native “soft” (PLL/HA)<sub>i</sub> multilayer coatings, where *i* is the number of layer pairs, were prepared on indium tin oxide (ITO) coated silicate glass slides with dimensions of 1x2 cm. Sequential layering of the polyelectrolytes and rinsing were done using robotized system Mindstorms (LEGO GmbH, Germany). First layer was adsorbed by 5 sec spraying of the PLL solution on ITO coated silicate glass slide. Then after 15 sec. pause

the PLL solution was rinsed by spraying of HEPES-2 buffer for 5 sec. Then HA was adsorbed and subsequently rinsed following the same procedure. Adsorption of PLL and HA was performed alternately until the desired multilayer was built. In some experiments, an additional layer of FN was adsorbed by immersion for 45 minutes. The multilayers were kept in 0.9% NaCl physiological solution (B-Braun AG, Germany).

### **Preparation of cross-linked [PLL/HA] multilayer substrates**

Cross-linked “semi-soft” (PLL/HA)<sub>i</sub> multilayer coatings, where *i* is the number of layer pairs, were prepared on indium tin oxide (ITO) coated silicate glass slides with dimensions of 1 x 2 cm.

Sequential layering of the polyelectrolytes and rinsing were done using robotized system Mindstorms (LEGO GmbH, Germany). First layer was adsorbed by 5 sec spraying of the PLL solution on ITO coated silicate glass slide. Then after 15 sec. pause the PLL solution was rinsed by spraying of HEPES-2 buffer for 5 sec. Then HA was adsorbed and subsequently rinsed following the same procedure. Adsorption of PLL and HA was performed alternately until the desired multilayer was built.

In some experiments, an additional layer of FN was adsorbed by immersion for 45 minutes.

Cross-linking of multilayers was performed with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) in combination with N-hydroxysulfosuccinimide sodium salt (NHS) similar as described by Richert et al [139]. The coupling chemistry is based on the reaction between activated carboxylate groups of HA and the ammonium groups of PLL and results in the formation of amide bonds. EDC and NHS were prepared in 0.9% NaCl in deionised water and mixed together immediately before use. The various EDC concentrations tested were 0, 8, 40, 80, and 800 mM; the NHS concentration was in all reactions 200 mM. The pH of mixed cross-linker solution was determined as 5.5. The multilayers were incubated in EDC/NHS for 12h at 4°C. Then the EDC/NHS solution was removed and the multilayers were rinsed three times with 0.9% NaCl physiological solution (1 x 1h, 1x 1h, 1 x 3h) to hydrolyse unreacted cross-linkers.

### **Preparation of [PAH-PSS] multilayer substrates**

Synthetic “stiff” (PAH/PSS)*i* multilayer coatings, where *i* is the number of layer pairs, were prepared on indium tin oxide (ITO) coated silicate glass slides with dimensions of 1x2 cm. Sequential layering of the polyelectrolytes and rinsing were done using robotized system Mindstorms (LEGO GmbH, Germany). First layer was adsorbed by 5 sec spraying of the PAH solution on ITO coated silicate glass slide. Then after 15 sec. pause the PAH solution was rinsed by spraying of HEPES-2 buffer for 5 sec. Then PSS was adsorbed and subsequently rinsed following the same procedure. Adsorption of PAH and PSS was performed alternately until the desired multilayer was built. In some experiments, an additional layer of FN was adsorbed by immersion for 45 minutes. The multilayers were kept in 0.9% NaCl physiological solution (B-Braun AG, Germany).

### **Validation of polyelectrolyte multilayer build-up by (OWLS)**

Deposition of polycations, polyanions and fibronectin on waveguides was measured *in situ* by OWLS as described by us and others previously [131, 138]. The instrument and the waveguides were obtained from (Micro Vacuum Ltd., Budapest, Hungary). The waveguide was mounted into a flow through cell with a volume of 15  $\mu$ L and left in HEPES-2 buffer overnight. After stabilization of the buffer baseline, polyelectrolyte solution was injected and let adsorbed for 5 min for PLL and HA, with 2 min rinsing steps in between. Adsorption of PLL and HA layers was performed alternately until the desired number of layers was built. In some experiments, an outer layer of FN was injected and let adsorbed for 120 min.

### 3.3. CELL SHEETS ON PEM SUBSTRATES: GROWTH AND CHARACTERIZATION

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*In the third part of the chapter I am describing materials and methods which were used for the assembling of PD-MSCs on the PEM coatings and their following characterization.*

#### 3.3.1. Cell attachment experiments

Cell attachment experiments were performed on PEM-coated, 1x2 cm silicate glass slides. AT-MSCs, PD-MSCs, and MBs were seeded at various cell densities  $0.5 \times 10^6$  cells/cm<sup>2</sup>, and cultured in non-hematopoietic stem cell culture medium (Miltenyi Biotec, Germany). Control cultures were grown on tissue culture plastic. For analysis of cell spreading, cytoskeleton was stained with rhodamine-labelled phalloidin according to the manufacturer's instructions (Invitrogen, Switzerland). Cell nuclei were stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR). Images were acquired with a DMIL fluorescence microscope (Leica) equipped with a DML digital camera (Leica) at the 10x, 20x or 40x magnification. Cell densities were quantified calorimetrically using crystal violet staining. For that, slides were fixed in methanol for 20 min, then transferred into new 12-well plates, and incubated with 0.5% crystal violet (AppliChem GmbH, Darmstadt, Germany) in 20% methanol for 1h at room temperature, then rinsed with water and air-dried. Destaining was performed by overnight incubation in 2% SDS. Optical density of the extracts was determined at 600 nm. In some experiments, cell counts were obtained from fluorescence micrographs of DAPI-stained cells using automated image analysis software Image J 1.34s (National Institute of Health, Bethesda, ML).

#### 3.3.2. Phenotypical characterization of MSC sheets

To analyse their phenotypical profile, PD-MSCs sheets were grown for 4 hours under standard cell culture conditions. After complete removal of the culture medium, cell sheets were washed once with PBS, and then dissociated by incubation with 0.25% Trypsin-EDTA solution (Invitrogen, Switzerland) at 37°C and 5%CO<sub>2</sub> for 5 min. For

FACS analysis  $10^5$  living cells per probe were prepared in custom made FACS buffer composed of PBS mixed with 1% of BSA. The cells were then incubated for 25 min at 4°C with fluorescently labelled primary antibodies HLA-ABC (BD Pharmingen, Switzerland), HLA-DR (BD Pharmingen, Switzerland), CD34 (Miltenyi Biotec GmbH, Germany), CD45 (Miltenyi Biotec GmbH, Germany), CD73 (BD Pharmingen, Switzerland), and unlabelled primary antibodies CD14 (BD Pharmingen, Switzerland), CD90 (BD Pharmingen, Switzerland), and CD105 (BD Pharmingen, Switzerland). Probes incubated with unlabelled primary antibodies were then additionally stained by incubation for 25 min at 4°C with a FITC labelled goat anti-human secondary antibody (BD Pharmingen, Switzerland). Unstained cells serve as negative controls. After staining all probes were washed with FACS buffer, fixed in 4% buffered formalin, and analysed with a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). A minimum of  $10^4$  gated events, were acquired for each sample.

### 3.3.3. Induced MSC differentiation on PEM substrates

Mesodermal plasticity of PD-MSC sheets was analysed by their induction with commercially available differentiation media, according the manufacture protocols (MACS guide). Precisely, PD-MSC cultures of passage 6 were plated either onto fibronectin-terminated, cross-linked (PLL/HA)<sub>9</sub>-PLL or native (PAH/PSS)<sub>9</sub> multilayers, deposited on ITO coated glass electrodes, at density  $5 \times 10^4$  cells per cm<sup>2</sup>.

#### **Adipogenic differentiation**

Adipogenic differentiation of PD-MSC sheets was conducted for 21 days. At day 21 induction medium was removed and cell sheets were fixed in ice-cold 100% methanol for 5 min at -20°C. After that cell sheets were washed once with deionised water lipid droplets were visualized by staining with 0.5% solution of Oil Red O (Sigma-Aldrich GmbH, Switzerland) for 20 min at RT. Finally, stained cell sheets were washed three times with water and analysed microscopically.

### **Chondrogenic differentiation**

Chondrogenic differentiation was initiated for 21 days. At day 21 induction medium was removed and cell sheets were fixed in 4% paraformaldehyde for 20 min at RT. After that cell sheets were washed once with PBS and collagen II expression was demonstrated by immunocytochemical staining with polyclonal goat anti-human collagen II antibodies (Santa Cruz, Ltd. USA) and secondary FITC-conjugated anti goat antibodies (DAKO Cytomation, Denmark). Cell nuclei were stained with 4'6- diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR). Finally, stained cell sheets were washed three times with PBS and analysed microscopically.

### **Osteogenic differentiation**

Osteogenic differentiation was initiated for 11 days. At day 11 induction medium was removed and cells were fixed in 70% ethanol for 40 minutes at RT. After that cell sheets were washed once with deionized water (B-Braun, Germany) and alkaline phosphatase expression was visualized by cytochemical staining with FAST BCIP/NBT (5-Bromo-4chloro-3-indolyl phosphate/Nitro Blue Tetrazolium) (Sigma-Aldrich GmbH, Switzerland). Finally, stained cell sheets were washed three times with water and analysed microscopically.

### **Confocal microscopy and image analysis**

Confocal images were acquired using Leica TCS-SP2 confocal microscope. The data were further processed using Imaris software v. 2.6 (Bitplane Scientific Solutions AG, Switzerland).

### **Immunocytochemistry**

For immunofluorescent staining, PD-MSCs or C2C12 cells were plated at density of  $1.5 \times 10^3$  cells/cm<sup>2</sup> on native or cross-linked FN-terminated [PLL/HA]<sub>9</sub> coatings, then cultured for 24h. Then cells were permeabilized with 0.1% Triton X-100, and stained for vinculin using fluorescein isothiocyanate (FITC)-conjugated anti-vinculin antibodies (1:100; Sigma-Aldrich Chemie GmbH, Switzerland); cell nuclei were stained with DAPI.

**Statistical analysis**

Data are shown as mean  $\pm$  SD. Two-tailed unpaired t test was performed using Graph Pad Prism version 4.00 for Windows (Graph Pad Software, San Diego, CA, USA). Significance level was set at  $p < 0.05$ .



### 3.4. CONTROLLED PEELING OF MSC SHEETS FROM PEM SUBSTRATES

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*In the fourth part of the chapter I am describing materials and methods which were used for the electrochemically and pH mediated peeling of the PD-MSC sheets as well as for the characterization of peeled PD-MSC sheets.*

#### **Spontaneously peeling**

Cells were plated out at density of  $1 \times 10^6$  cells/cm<sup>2</sup> on “stiff” [PAH-PSS]<sub>9</sub> coating and cultured for 12 h. After reaching confluence spontaneously peeling of cell sheets was started and they were mechanically removed from the PEM coating using tweezers. Resulting cell sheets were then analysed for the viability using live-dead staining. Additionally, peeled cell sheets were plated out in fibronectin coated six well plates and differentiated towards osteogenesis, chondrogenesis, and adipogenesis using standard differentiation conditions has been described before.

#### **pH mediated peeling**

For pH mediated cell sheet peeling NH expansion media with pH = 4, 5, 6, and 7 have been prepared. The pH adjustment was done using 1M NaOH and 6M HCl, respectively. After adjustment, pH was controlled using Seven Easy pH measure machine (Mettler Toledo GmbH, Switzerland). Cells were plated out at density of  $0.5 \times 10^6$  cells/cm<sup>2</sup> on “stiff” [PAH-PSS]<sub>9</sub> coating and cultured for 12 h. After that, NH expansion medium has been removed and confluent cell sheets were washed once with 1 x PBS. NH expansion medium with different adjusted pH has been applied to the [PAH-PSS]<sub>9</sub> coated ITO silicate glass surfaces for 15 min at RT. Peeled at pH = 4 cell sheets have been analysed for vitality using live-dead staining procedure as described before.

#### **Peeling mediated by electrochemical means**

For electrochemically-induced cell sheet detachment, PD-MSCs were cultured for 4 hours on (PAH/PSS)<sub>9</sub>-coated ITO glass chips. The chips were then mounted in a custom made electrochemical flowcell provided with a similar three-electrode configuration

system as described before. Non-hematopoietic stem cell expansion media (Miltenyi Biotec GmbH, Germany) was supplemented with 3 mM HEPES. A current at density 30  $\mu\text{A}/\text{cm}^2$  was applied using an AMEL potentiostat/galvanostat (model 2053, AMEL electrochemistry, Italy) and the samples were observed by phase contrast microscopy (DM IL, Leica Microsystems Ltd, Switzerland) until complete cell sheet detachment.

### **Live-dead staining of harvested stem cell sheets**

Harvested PD-MSC sheets were analysed for vitality using live-dead staining procedure. For this cell sheets were rinsed three times with PBS and then incubated with a mixture of 1  $\mu\text{M}$  calcein acetate and 2  $\mu\text{g}/\text{ml}$  ethidium homodimer for 30 min. After rinsing three times with PBS, images were acquired using Zeiss Axiovert 200M (Carl Zeiss, Switzerland) equipped with a digital camera AxioCam MRc (Carl Zeiss, Switzerland), and finally analysed with AxioVision V. 4.5.0.0 software (Carl Zeiss, Switzerland).

### 3.5. INSTRUCTED DIFFERENTIATION OF MSC SHEETS AFTER PEELING

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*In the fifth part of the chapter I am describing materials and methods which were used for the instructed differentiation of the electrochemically peeled PD-MSC sheets.*

#### **Mesodermal differentiation of harvested stem cell sheets**

Peeled PD-MSC sheets were transferred to Petri dishes with diameter 35 mm (TPP, Switzerland) and allowed to adhere in non-hematopoietic stem cell expansion medium (Miltenyi Biotec GmbH, Germany) at 37°C and 5% CO<sub>2</sub> for 24 hours. After that culture medium was replaced with AdipoDiff, ChondroDiff, and OsteoDiff induction media (Miltenyi Biotec GmbH, Germany), respectively. Appropriate differentiation and staining procedures were performed according the manufacture protocols as described before.

#### **Co-culture of MSC sheets with osteoblasts - indirect contact**

Human osteoblasts (HOB) were plated out at density  $0.5 \times 10^6$  cells/cm<sup>2</sup> in the 12 well/plate (TPP, Switzerland). Cells were grown to confluence in non-hematopoietic stem cell expansion medium (Miltenyi Biotec GmbH, Germany) at 37°C and 5% CO<sub>2</sub>. At day 7 peeled PD-MSC sheets were transferred into the insert wells and co-cultured with HOB cultures for 14 days. In differentiation conditions PD-MSC sheets co-cultured with HOB cultures were additionally pre-stimulated with OsteoDiff medium (Miltenyi Biotec GmbH). In the co-culture control conditions PD-MSC sheets were co-cultured with PD-MSC cultures and pre-stimulated with OsteoDiff medium. In the negative control conditions PD-MSC sheets were co-cultured with PD-MSC cultures without pre-stimulation with OsteoDiff medium. At day 15 insert wells with cell sheets were removed and alkaline phosphatase expression was visualized by cytochemical staining with FAST BCIP/NBT (5-Bromo-4chloro-3-indolyl phosphate/Nitro Blue Tetrazolium) (Sigma-Aldrich GmbH, Switzerland). Finally, stained cell sheets were washed three times with water and analysed microscopically. Additionally, collagen I expression was analysed using Real-Time PCR.

### **Co-culture of MSC sheets with osteoblasts - direct contact**

Human osteoblasts (HOB) were plated out at density  $0.5 \times 10^6$  cells/cm<sup>2</sup> in the 12 well/plate (TPP, Switzerland). Cells were grown to confluence in non-hematopoietic stem cell expansion medium (Miltenyi Biotec GmbH, Germany) at 37°C and 5% CO<sub>2</sub>. At day 7 peeled PD-MSC sheets were transferred direct on the layer of HOB cells and co-cultured for 14 days as described above. At day 15 PD-MSC sheets were removed from the plate using scalpel and collagen I expression was determined using Real-Time PCR.

### **Real-Time PCR**

Acquisition was done using Step One Plus Real-Time PCR system (Applied Biosystems, CA). Analysis of results was done using Step One Software v. 2.1 (Applied Biosystems, CA). All preparation steps as well as analytical procedures were done according the manufacture protocol.

# ISOLATION OF PLACENTAL MSCs

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*This chapter of the thesis is published as per-reviewed article entitled as “Multipotent mesenchymal stem cells from human term placenta: critical parameters for the isolation and maintenance of stemness after isolation” (AJOG 2010; 202: 193.e1-13). Stem cell isolations were done in cooperation with Dr. Antoine Malek. Immunocytochemical analysis was performed by Dr. Sonya Koestenbauer.*

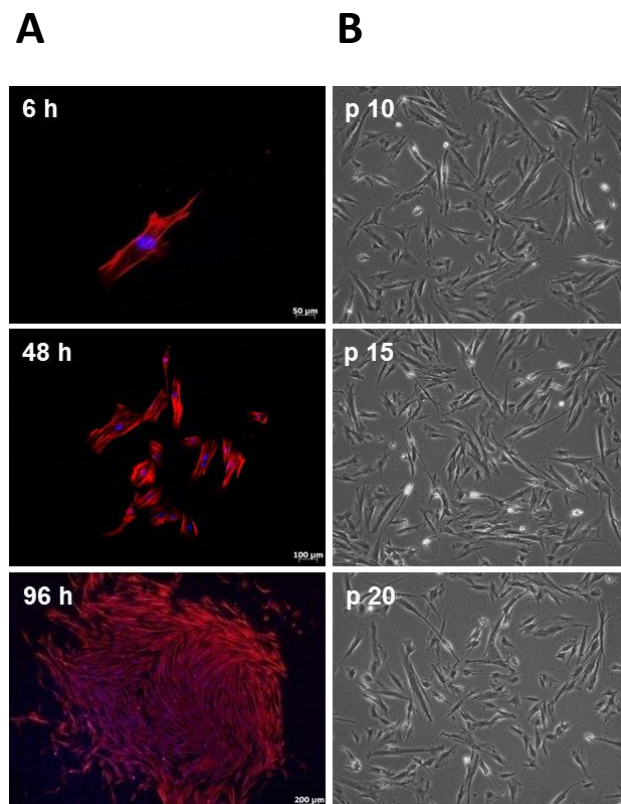
### 4.1. Potential of mesenchymal stem cells for regenerative medicine

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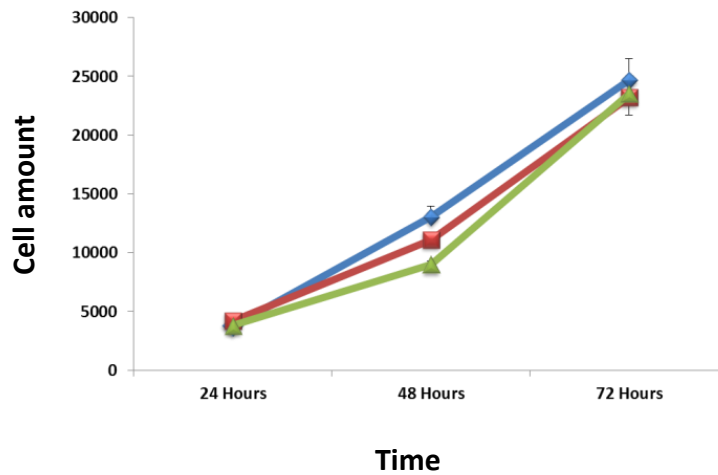
As was mentioned in the introduction mesenchymal stem cells (MSCs) represent an interesting cell type for research and therapy because of their ability to differentiate into mesodermal lineage cells, such as osteocytes, chondrocytes, cardiac muscle, or endothelial cells [141]. In addition, they secrete large amounts of proangiogenic and anti-apoptotic cytokines and possess remarkable immunosuppressive properties [142, 143]. Mesenchymal stem cells have been derived from many different organs and tissues [144]. Evidence has emerged that also different parts of human placenta, umbilical cord, and amniotic membrane, as well as umbilical cord blood, harbour MSCs [105, 145-148]. These tissues are normally discarded after birth, avoiding ethical concerns [149]. Mechanical, as well as enzymatic, methods for MSC isolation from different regions of human placenta of different gestational ages were reported [106, 141, 148, 150-163]. Knowledge about vitality, karyotype, phenotype, and expandability of such placenta-derived MSC isolates is a prerequisite for therapeutic application; however, systematic investigations into reliability of this MSC source and phenotypic stability have not yet been attempted. Furthermore, former reports on placenta-derived MSCs often lack information about the karyotype of the cell isolates. In this chapter, I described enzymatic fractionation of term human placenta that allows recovery of multipotent, fibroblast-like cells, which were then used for engineering of stem cell sheets as described in the subsequent chapters. I tentatively term these cells as placenta-derived mesenchymal stem cells (PD-MSCs) with high fidelity.

## 4.2. Isolation and characterization of MSCs from human term placenta

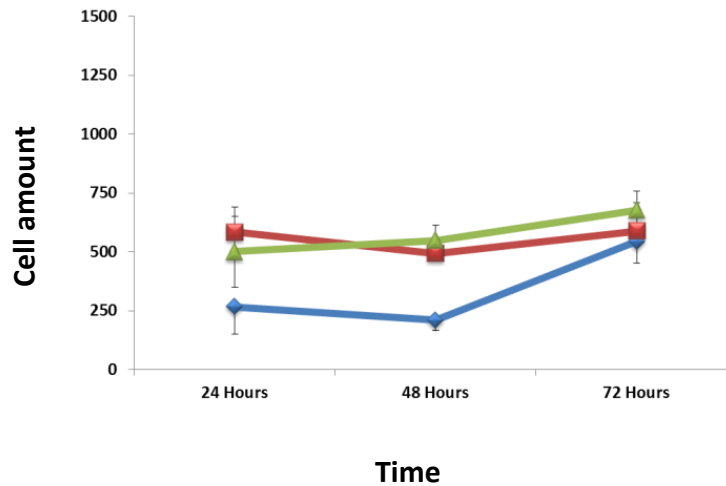
The flow chart of the enzyme-mediated fractionation of human term placenta for derivation of fibroblast-like cells, which I tentatively term placenta-derived multipotent mesenchymal stem cells (PD-MSCs), is depicted in **[Figure 24]**. Selection for MSCs rested on the classic adhesion method on tissue culture plastic. The protocol proved successful in 14 of 17 test cases. Cell suspensions produced from final collagenase I digest of approximately 15 g placental tissue fragments typically produced 300–500 colonies of variable sizes that contained outgrowing fibroblast-like cells. Typically, approximately  $4\text{--}6 \times 10^4$  cells were obtained within 2 weeks after plating. Plating of cell suspensions from the first digest with trypsin did not produce any colonies. Outgrowing cells when harvested and plated again in high dilution rapidly formed secondary colonies from single cells **[Figure 25, A]**. Cultures of PD-MSCs were expandable up to passage 20 (as far as we cultured) without change of fibroblast-like morphology **[Figure 25, B]** or proliferation rate **[Figure 25, C]**.



**C**



**D**

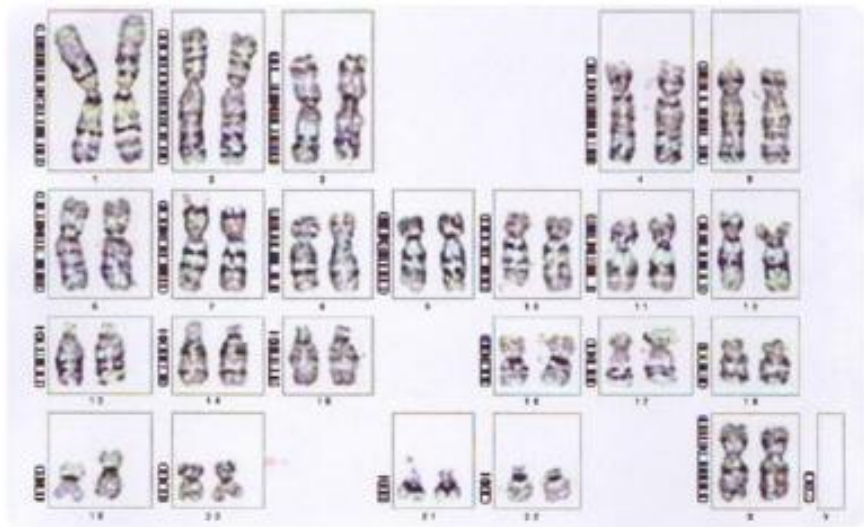


**Figure 25: Morphology and growth kinetics of PD-MSCs. (A) Secondary colony formation from single cells. Cell shape and organization were analysed by fluorescence microscopy to visualize phalloidin stained F-actin cytoskeleton (red) and DAPI stained cell nuclei (blue). Phase microscopic images of PD-MSCs at indicated passages (B). Growth kinetics of PD-MSCs at indicated passages (C). Mortality rates at indicated passages (D) Asterisk indicates significant enhancement ( $P < .05$ ) of cell numbers between time points.**

Chromosomal analysis of cell isolates from 6 different placental cases (3 of female and 3 of male deliveries) did not show any chromosomal abnormalities [Figure 26].



Karyotypes were normal 46, XX in all test samples, including the 3 PD-MSC isolates obtained from male deliveries **[Table 2]**.

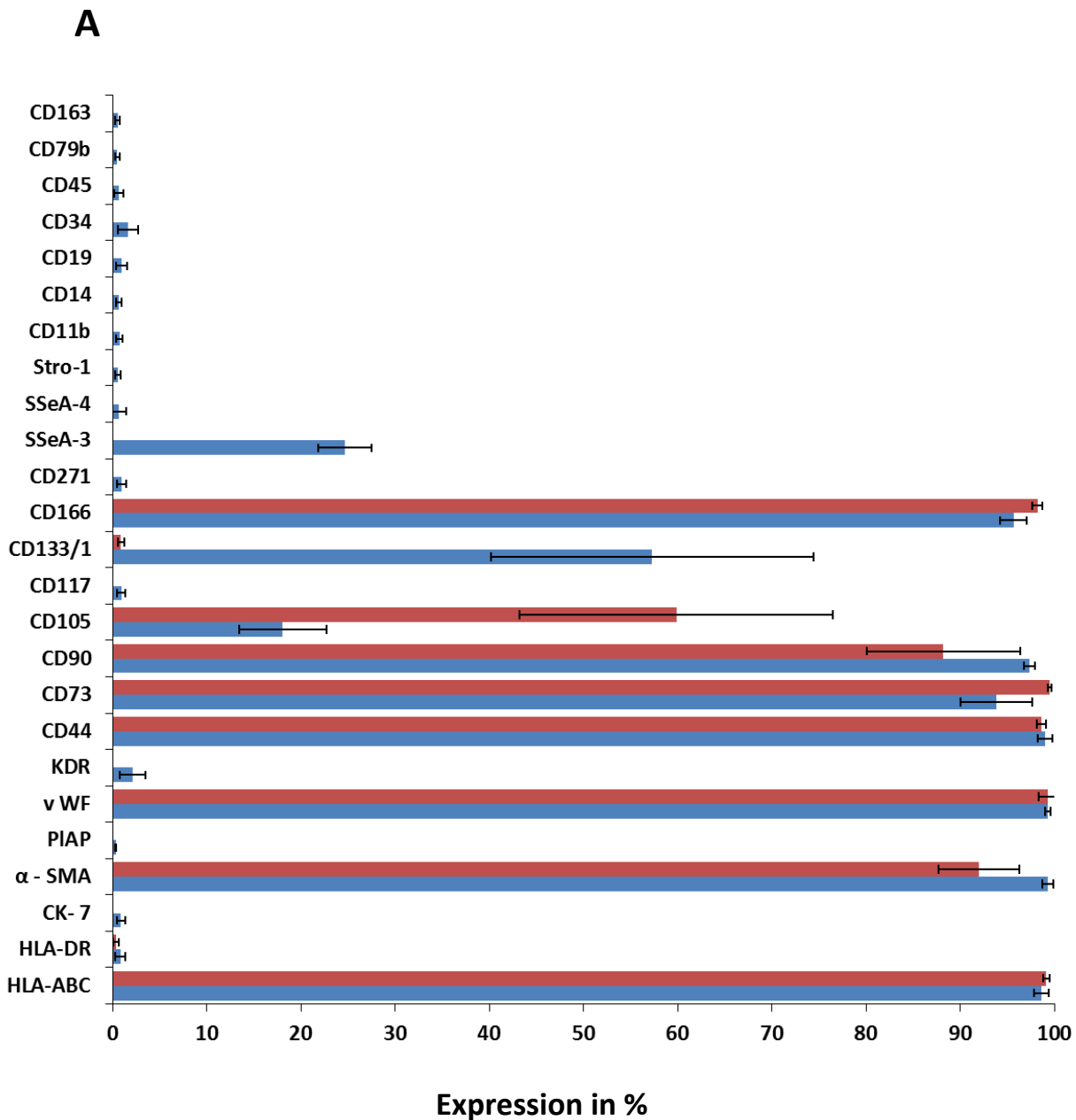


**Figure 26: Representative example of chromosome analysis of PD-MSC cultures at passage 3 (n=6)**

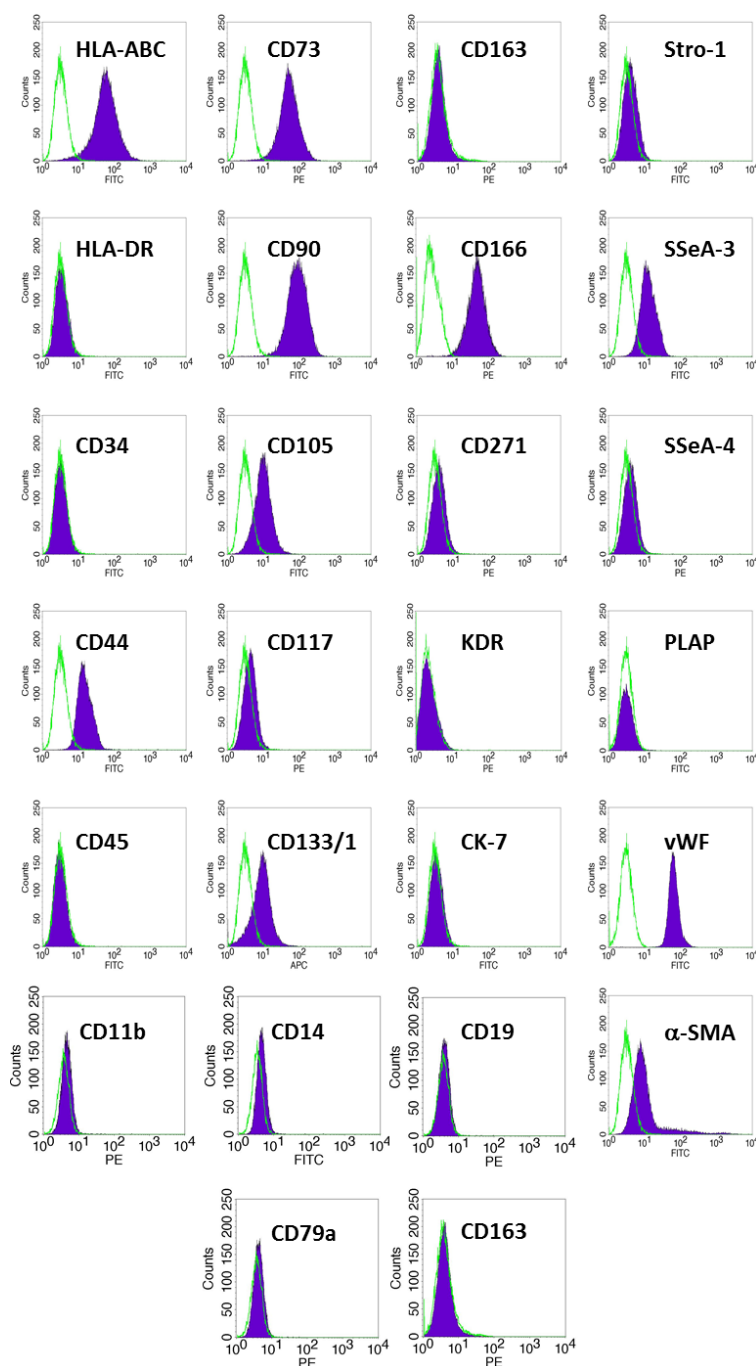
DONOR	FOETUS	NORMALITY	KARYOTYPE
2	Female	Normal	46 XX
4	Female	Normal	46 XX
7	Female	Normal	46 XX
9	Female	Normal	46 XX
11	Female	Normal	46 XX
13	Female	Normal	46 XX

**Table 2: Cytogenic analysis of PD-MSCs. Data represent karyotyping of passage 3 cell isolates obtained from three female and three male deliveries.**

FISH analysis for the SRY gene confirmed the absence of male cells in our PD-MSC isolates. Thus, our procedure isolated only PD-MSCs of maternal origin, which was unexpected. The phenotype of the cell isolates, passage 1, was examined by flow cytometry for 5 subjects [Figure 27] and further examined by immunocytochemistry with a panel of antibodies against 30 antigens for a single subject [Figure 28].



**B**



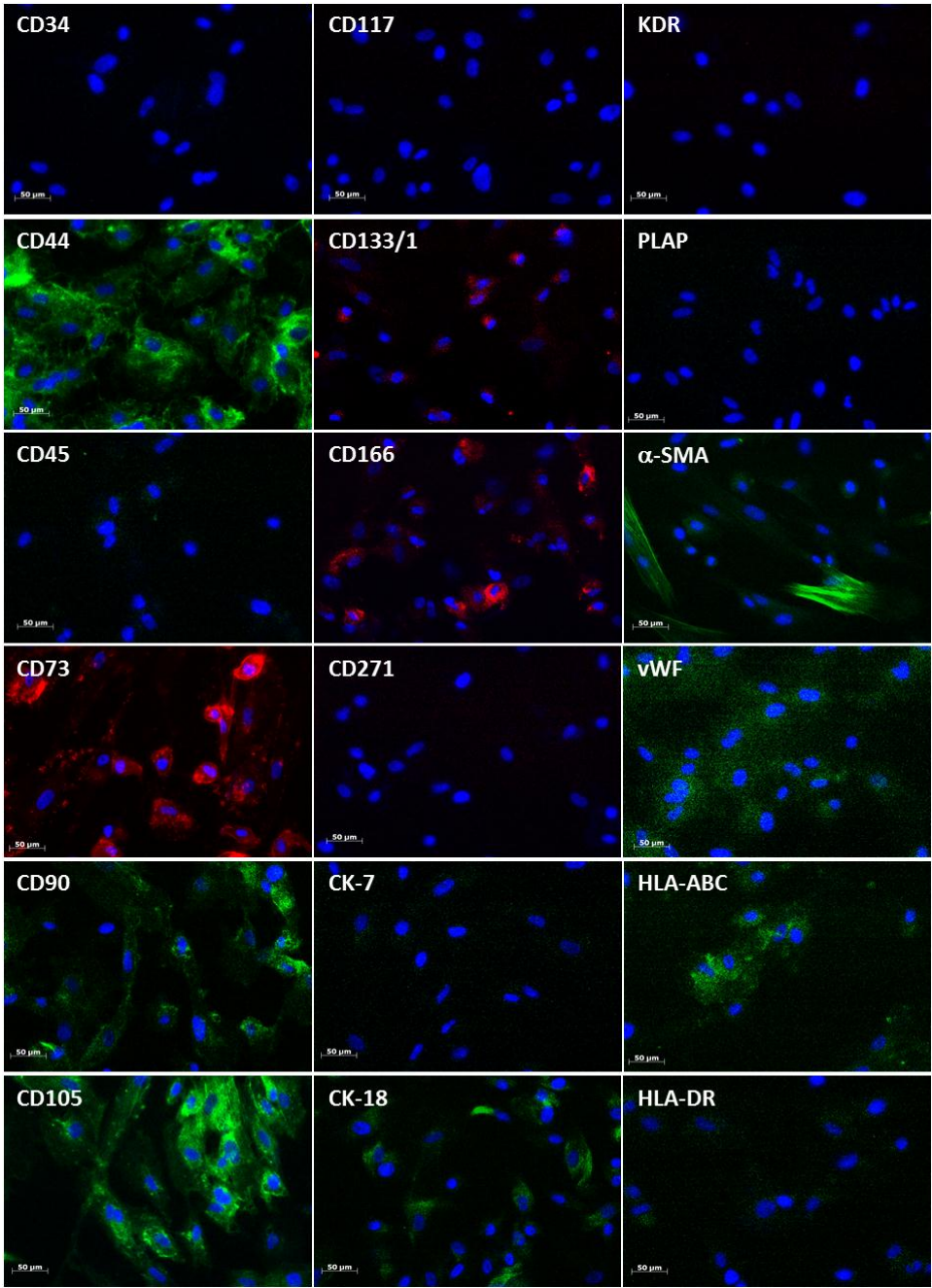
**Figure 27: PD-MSC phenotype at passages 1 and 20 as determined by flow cytometry. Screen of PD-MSC isolates at passage 1 (n = 5) shown in blue colour, and passage 20 (n = 5) shown in red colour (A). Representative example of PD-MSC isolates at passage 1 (B). Green lines denote unstained control cells.**

Flow cytometry revealed very little scatter in the phenotypic marker profile of placenta-derived isolates between cases. The expression profile conformed to the criteria recently defined for multipotent mesenchymal stem cells **[Figures 27 A,B]** [164]. Cell isolates were uniformly positive for mesenchymal stem cell markers CD44, CD73, CD90, CD105, and CD166. Hematopoietic markers CD34 and CD45, as well as CD11b, CD14, CD19, and CD79-alpha, were not expressed. Cells stained also negative for macrophage-restricted antigen CD163, which marks Hofbauer cells, the cytotrophoblast-specific marker Cytokeratin and syncytiotrophoblast-specific marker placental alkaline phosphatase [147, 165, 166]. Curiously, the entire cell population was positive for 2 markers of end-differentiated cells, such as the endothelial protein vWF, and the smooth-muscle cell protein-SMA. Isolates of PD-MSCs were negative for kinase insert domain-containing receptor, Cytokeratin 18, CD117, and CD271. We found high levels of human leukocyte antigen (HLA)-ABC (MHC class I cell surface receptor) but no HLA-DR (MHC class II cell surface receptor). Both flow cytometry and immunocytochemistry revealed that part of the passage 1 cells expressed the antigen CD133, which is considered as a marker of non-committed early progenitors of blood cells, endothelial cells, and other stem cell types [167, 168]. Immunocytochemistry did not detect expression of human stem cell markers of pluripotency, such as the transcription-binding domains Oct3/4, Stro-1, Tra-1-60, or Tra-1-81. Flow cytometry and immunocytochemistry revealed that PD-MSCs were positive for stage-specific embryonic antigen SSeA-3 but negative for SSeA-4 **[Table 3 and Figures 28]**.

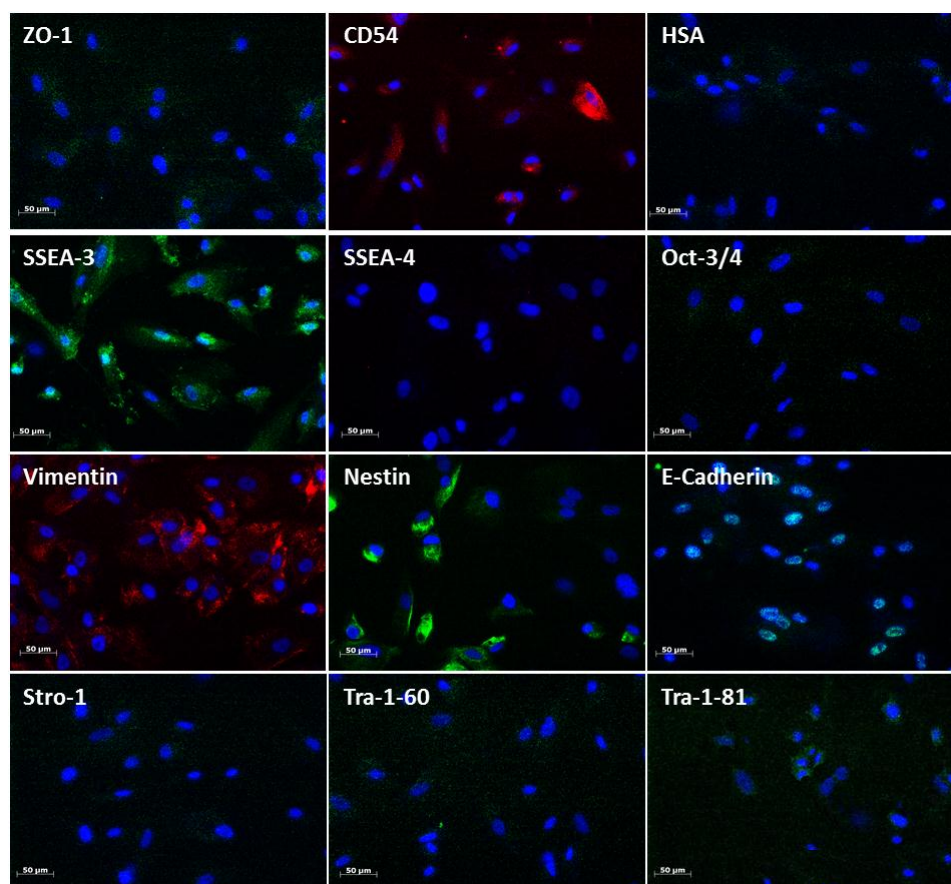
Specific induction of differentiation was investigated with PD-MSCs of passage 6, from 3 subjects. This confirmed that the mesenchymal stemness profile by PD-MSC populations indeed associated with the ability to generate different mesodermal lineage cell types on their exposure to soluble factors *in vitro* [169]. Representative results of adipogenic **[Figure 29, A and B]**, chondrogenic **[Figure 29, C and D]**, and osteogenic **[Figure 29, E and F]** differentiation assays, visualizing lipid vacuoles, chondrogenic matrix, and calcium deposits, respectively. Moreover, culture in presence of the angiogenic growth factor VEGF induced expression of marker CD34, which is a marker of hematopoietic, as well as endothelial, precursors **[Figure 29, G and H]**

[170]. The latter finding points to a broader mesodermal differentiation capacity of PD-  
MSCs.

A

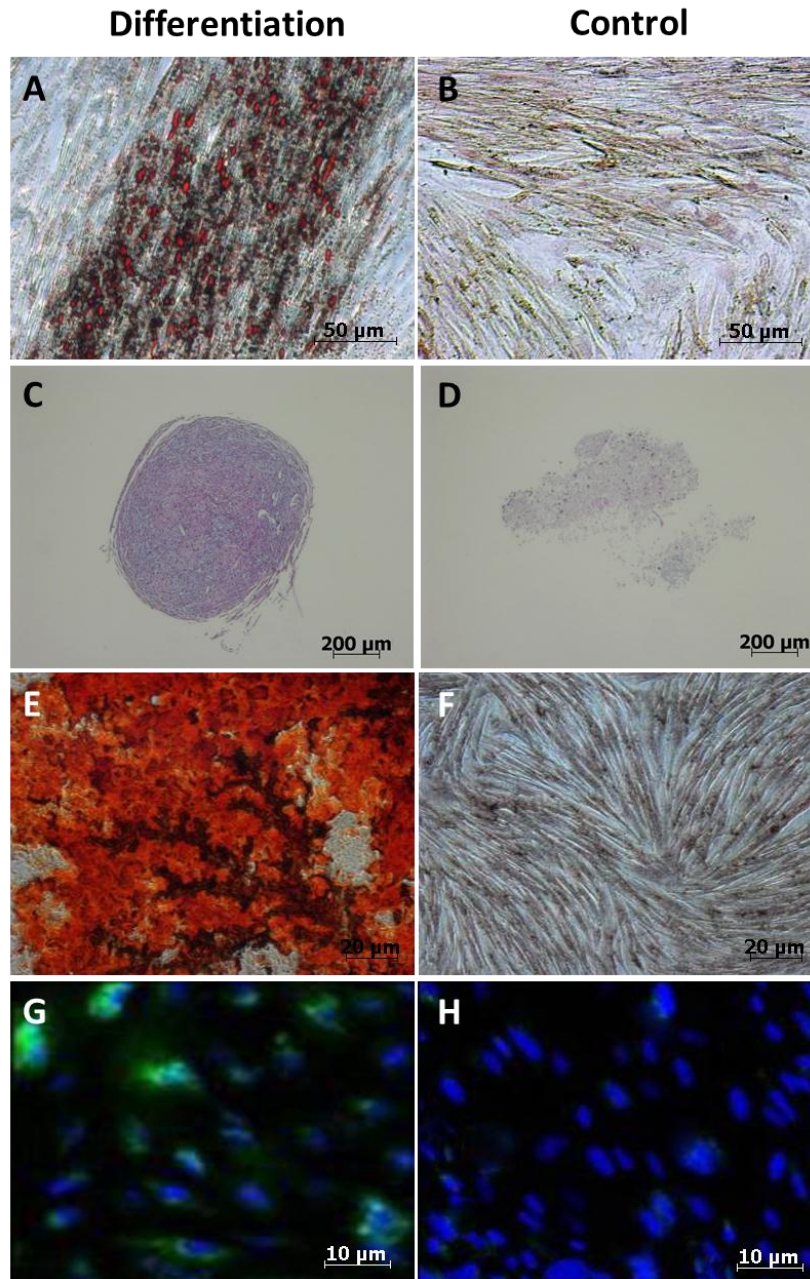


**Figure 28: PD-MSC phenotype at passage 6 (n=1) as determined by immunocytochemistry.**



**Figure 28: PD-MSC phenotype at passage 6 (n=1) as determined by immunocytochemistry.**





**Figure 29:** A representative differentiation of PD-MSCs, passage 6, is shown. Cells were kept in induction medium (differentiation) or maintained without induction in standard medium (control). Adipogenic differentiation and control as determined by Oil-Red-O staining of lipid droplets (A, B); chondrogenic differentiation as determined by Alcian Blue staining of proteoglycans (C, D); osteogenic differentiation, as determined by Alizarin Red S staining of calcium deposition (E, F); endothelial differentiation and control, as determined by CD34 expression in green (G, H).

ANTIGEN	FACS	IC
CD11b	-	N.D.
CD14	-	N.D.
CD19	-	N.D.
CD34	-	-
CD44	-	+
CD45	-	-
CD54	N.D.	20%
CD73	+	+
CD79a	-	N.D.
CD90	+	+
CD105	+	30%
CD117	-	-
CD133/1	+	33%
CD163	-	N.D.
CD166	+	+
CD271	-	-
Cytokeratin-7	-	N.D.
Cytokeratin-18	N.D.	40%
VEGF receptor / KDR	-	-
Placental Alkaline Phosphatase	-	-
$\alpha$ -Smooth Muscle Actin	+	+
Von Willebrandt Factor	+	+
Hepatocyte Specific Factor	N.D.	-
HLA-ABC	+	+
HLA-DR	-	-
SSEA-3	+	+
SSEA-4	-	-
Oct-3/4	N.D.	-
Vimentin	N.D.	+
Nestin	N.D.	50%
E-Cadherin	N.D.	40%
ESCM Tra-1-60	N.D.	-
ESCM Tra-1-81	N.D.	-
ZO-1	N.D.	-

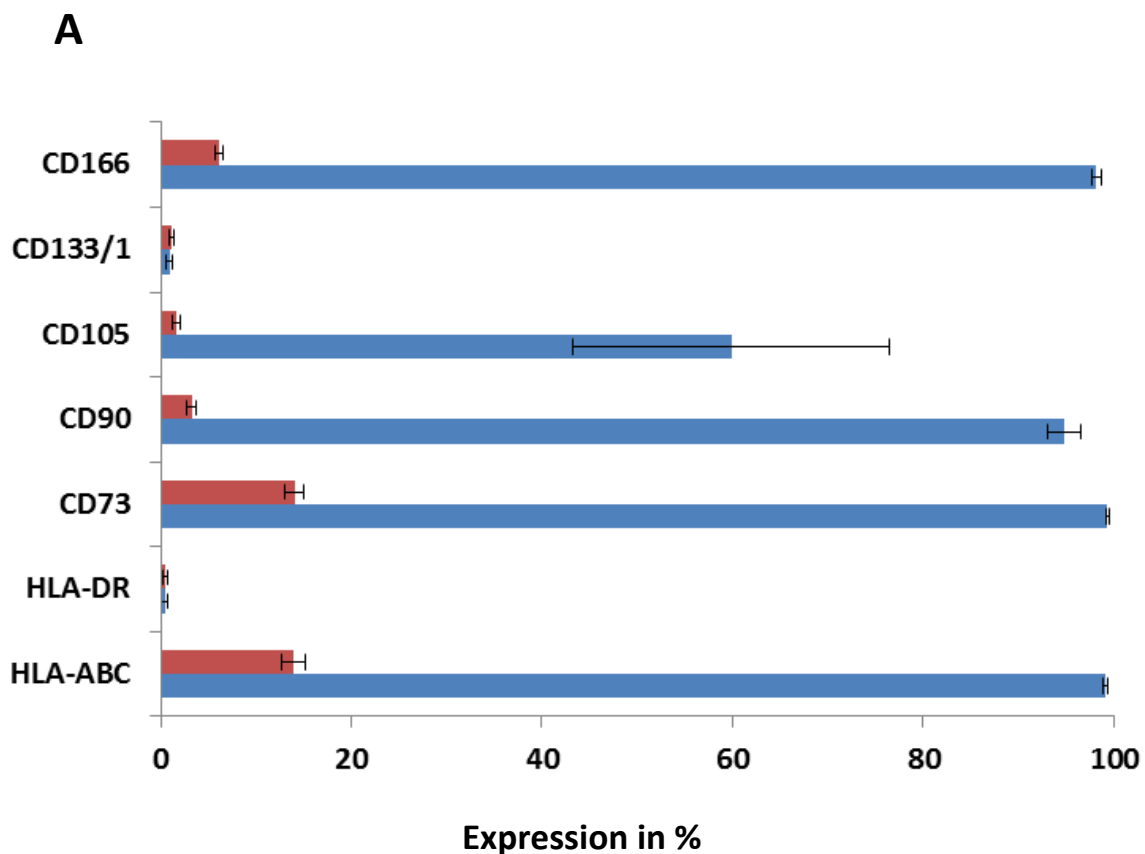
**Table 3: Phenotypic analysis of PD-MSCs by flow cytometry and immunocytochemistry**

#### 4.3. Long term cultivation and differentiation of placental MSCs

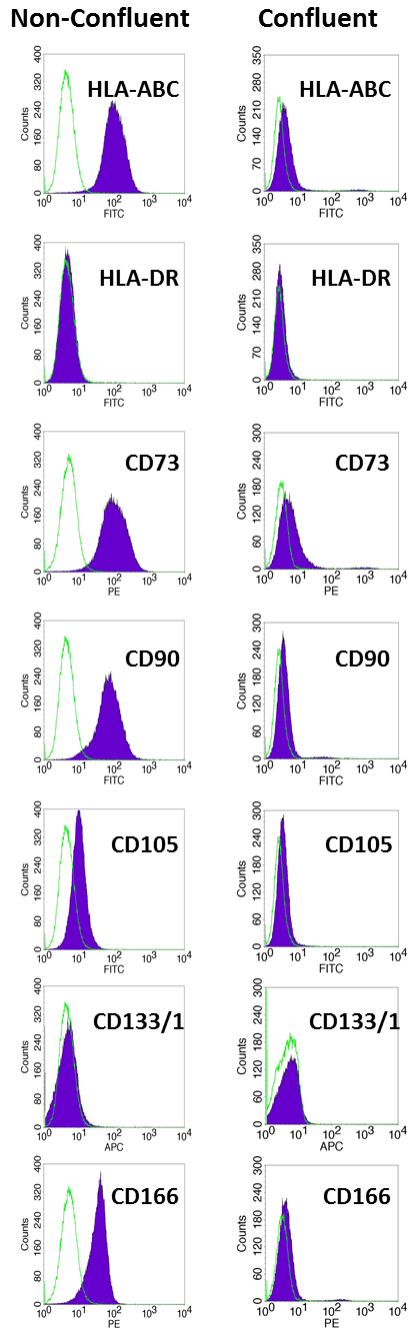
Sub-confluent culture was found critically important to maintain the stemness phenotype of PD-MSCs during expansion. The results of a side-by-side screen for marker profiles of PD-MSCs that were grown at confluent versus sub-confluent culture



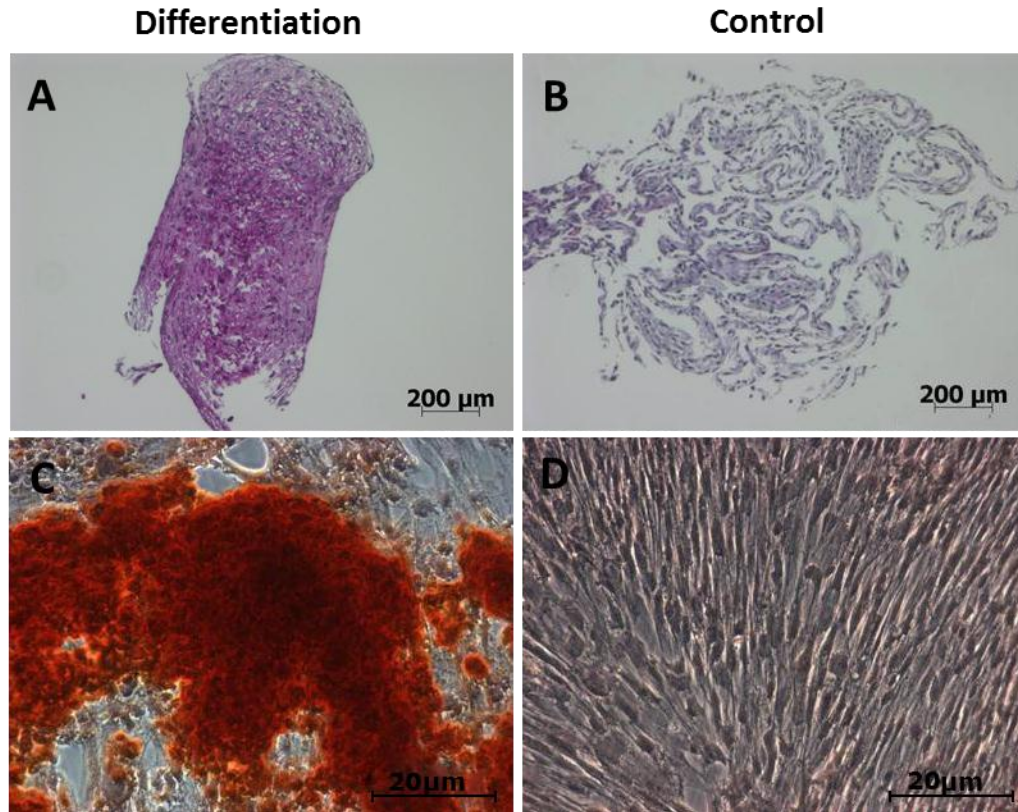
conditions until passage 10 are depicted on the **[Figure 30]**. The screen was performed with 4 cases of each condition. The phenotypic profile of PD-MSCs when sub-cultured at 50–70% cell density remained unaffected. In sharp contrast, PD-MSCs grown in confluent or near confluent culture lost mesenchymal stem cell markers almost completely. Expression of CD133 antigen disappeared during culture in both conditions. PD-MSCs passaged in sub-confluent culture maintained their initial marker profile and their ability to differentiate as well. A direct comparison of the marker profiles of passage 1 PD-MSCs versus passage 20 PD-MSCs is depicted on the **[Figure 27]**. Passage 20 PD-MSCs could be induced toward chondrogenic and osteogenic lineage *in vitro*, as demonstrated by staining of chondrogenic matrix and calcium deposits, respectively **[Figure 31]**, whereas adipogenic differentiation failed (data not shown).



**B**



**Figure 30: PD-MSC phenotype in sub-confluent versus confluent subculture. Screen of PD-MSC isolates, passage 10 (n=5). Sub-confluent (blue) culture was found critically important to maintain MSC marker expression (A). Representative example of PD-MSC isolate at passage 10. (B)**



**Figure 31:** Cells were kept in induction medium (differentiation) or maintained without induction in standard medium (control). Chondrogenic differentiation and control, as determined by Alcian Blue staining of proteoglycans (A, B); osteogenic differentiation and control, as determined by Alizarin Red S staining of calcium deposition (C, D).

#### 4.4. Placenta-derived mesenchymal stem cells for cell sheet engineering

In demonstrating that term human placenta, which is a readily available and ethically relatively unproblematic tissue, constitutes a robust source of MSCs, we investigated several parameters - namely, (I) chromosome number, (II) origin, (III) methods of isolation, and (IV) propagation - that are important for their principal utility for cell-based therapy and could influence their proliferative, as well as differentiation, capacities. Chromosome number was found normal in all analysed PD-MSC isolates (n=6). Looking at maternal or foetal origin, we found that PD-MSC isolates obtained with our isolation procedure were always of maternal origin, which was contrary to our expectations. This finding corroborates the very recent observation by Barlow et al. that

cells isolated from placentas of male delivered babies were not of foetal origin [163]. In contrast, the study by Fukuchi et al., which is the only investigation, reported the detection of foetal origin cells isolated from human term placenta [154]. However, looking at the image provided in the same study, a fraction of the isolated cells was of foetal origin, whereas the remaining cells were of maternal origin.

The precise placental anatomic origin of the maternal cells obtained with our procedure is still unclear. One source could be maternal decidua, despite our careful attempts to remove this layer completely before digestion of placenta. Placental septa constitute another potential source. One explanation for the obvious difficulty to capture foetal mesenchymal stem cells from term human placenta is offered by the fact that most stem cells observed in the first trimester of pregnancy are differentiated to cytotrophoblast and endothelium cells in terminal and intermediate villi, whereas the few remaining stem cells were detectable only around the stem villi [171, 172]. Furthermore, the thinning of the stromal layer and decreased cell density toward the end of gestation is known as a positive adaptation to ensure the increasing nutritional supply of the growing foetus [173]. We think that the most likely origin of the maternal cells obtained in our study is decidual tissue that remained on placental septa, which is difficult to remove. Our method of cell isolation by way of sequential digestion of the trophoblast cell layer with trypsin and following digestion of remaining placental tissue with collagenase I proved very effective for obtaining PD-MSCs. Outgrowth of PD-MSCs from collagenase digests was successful in 14 of 17 test subjects and resulted in populations with remarkably little scatter in their MSC profiles, between subjects. As for propagation, we found out that PD-MSCs must be propagated in sub-confluent culture to maintain their MSC profile, because confluent culture led to gradual loss of MSC identity. With proper sub-confluent passage, PD-MSCs maintained their phenotypic MSC profile up to the highest passage number we tested in this study, passage 20, as well as their differentiation capacity. As detailed above cultures of PD-MSCs, after 1 passage, were constituted by a homogenous population of fibroblast-like cells, which expressed the typical mesenchymal stem cell markers but showed no contamination with macrophages, hematopoietic cells, or trophoblast cells **[Figure 27]**. We observed by flow cytometry and immunocytochemistry that early passage cultures expressed

CD133, a protein of unknown functionality that is considered a marker of immature cells. CD133 expression has been found restricted to stem cells in normal adult tissue and expressed by non-committed early progenitors of blood cells and endothelial cells, non-malignant neural progenitors, but also as tumour-initiating stem cells in brain [167, 174, 175]. Recent studies showed that CD133-positive cell fractions of mononuclear cell human peripheral blood and cord blood, which were isolated by magnetic sorting with micro-beads coated with anti-CD133 antibody, give rise to MSCs [168]. Magnetic sorting of CD133-positive cells from placental extracts, therefore, could provide an alternative to MSC selection by adhesion to tissue culture plastic. The growth potential of PD-MSCs, as assessed by their growth kinetics at passages 1, 10, and 20, was unaffected by subculture. Furthermore, our studies of stemness in functional differentiation assays showed that PD-MSCs retain their ability to generate chondrocytes and osteocytes at passage 20, although adipogenic potential was obviously lost at late passage. Induction with VEGF of endothelial differentiation in early-passage PD-MSCs showed that the induced cells expressed CD34, a marker for endothelial precursors. We strongly caution about an interpretation that PD-MSCs could differentiate into endothelial cells but rather believe that this finding could point to a broader plasticity of PD-MSCs.

#### 4.5. Achievements of the fourth chapter

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In the first part of my thesis I developed a highly reproducible, straightforward methodology that leads to isolation of multipotent mesenchymal stem cells of maternal origin from human term placentas. These cells were then used for generation of stem cell sheets. Further studies in this direction are indicated to evaluate the properties of such maternally derived PD-MSCs regarding their full differentiation capacity *in vitro* and ultimately *in vivo*. Aside from evaluating their utility for future cell replacement therapy and tissue engineering, we seek to resolve whether such PD-MSC isolates could become exploited in transplantation medicine for an ability to exert immunosuppressive effects and/or to secrete proangiogenic and antiapoptotic cytokines to aid tissue healing [142, 176].

## CHAPTER 5

# STEM CELL SHEET ENGINEERING ON PEM SUBSTATES

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*This chapter of the thesis is published as per-reviewed article entitled as “Engineered polyelectrolyte multilayer substrates for adhesion, proliferation, and differentiation of human placental mesenchymal stem cells. (TISSUE ENGINEERING Part A Volume 15, Number 10, 2009). Confocal microscopic analysis was performed in cooperation with Dr. Anne-Greet Bittermann.*

### 5.1. Polyelectrolyte multilayer (PEM) substrates

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Polyelectrolyte multilayer assemblies (PEM) can be generated from very broad range of natural and synthetic materials. Among them are: poly amino acids (poly-L-lysine (PLL), poly-L-(glutamic acid)), polysaccharides (hyaluronic acid (HA), dextran sulphate, and chitosan), or synthetic polymers (poly (allylamine hydrochloride), poly-methacrylic acid, or sulfonated poly-(styrene)) [177]. Adsorbent species, time of adsorption, and concentration of adsorbed polyelectrolytes but, importantly, also the conditions under which PEMs are built up (pH, rinsing solution, ionic strength, number of layering steps, assembly order, chemical cross-linking) dictate the morphology, thickness, and internal structure of the coatings, which in turn determine their distinctive bioactivity. Coatings assembled from PEM with highly adjustable bioactive properties were demonstrated. For example, varying the build-up pH or ionic strength directed single multilayer combinations to be adhesive versus resistant surfaces for fibroblasts or to be anti-versus pro-coagulate surfaces [177, 178]. Coatings built from PEM can also serve as reservoirs. Various compounds such as DNA, RNA, drugs, dyes, inorganic particles, proteins, and peptides can be embedded in PEMs or deposited on top of them. For embedded peptide hormones or antibodies, the biological activities were found to be retained [139, 179, 180]. Several studies have recently investigated cell interactions with PEMs. The biophysically and biologically best-studied systems are arguably multilayer coatings of anionic HA and cationic PLL [132, 181, 182]. Even micrometre-thick films of (PLL/HA)*i* have been achieved, where *i* is the number of layer pairs [183]. HA is a natural, linear polysaccharide with repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with  $\beta$  (1P4) interglycosidic linkage and is found in the extracellular matrix

of connective tissue, where it is present as a hydrated network with collagen fibres. Its average molar mass ranges from approximately  $10^5$  to  $10^7$  Da. HA provides excellent biocompatibility, and native and modified HA have been used for ophthalmic surgery, arthritis treatment, drug delivery, and tissue engineering [184]. The counter polycation PLL is widely used in cell culture to promote cell adhesion to solid substrates and can be easily conjugated with bioactive molecules. Mechanistic studies indicated that (PLL/HA) multilayer build-up begins with isolated islands that grow by addition of new polyelectrolytes on their top and by mutual coalescence and that continuous films are obtained after the eighth layer pair deposition [132]. Native, as-built PEM coatings are soft, elastic substrates that did not support cell adhesion well. Increasing stiffness of (PLL/HA) multilayers was most influential for cell adhesion [139]. Using chemical cross-linking of (PLL/HA) multilayers by water-soluble carbodiimide in combination with N-hydroxysulfosuccinimide coatings were obtained with an effective Young's modulus that was more than 10 times as large as in a native, non-cross-linked multilayer substrate [182]. Tests of cross-linked (PLL/HA) multilayer substrates with primary chondrocytes and smooth muscle cells showed greater cell anchoring and spread on the cross-linked layers [139, 185].

As was mentioned in the first part of the thesis, mesenchymal stromal cells (MSCs) represent a particularly interesting cell type for research and therapy because of their ability to differentiate into mesodermal lineage cells such as osteocytes, chondrocytes, cardiac muscle, and endothelial cells [154]. In addition, they secrete large amounts of pro-angiogenic and anti-apoptotic cytokines, and possess remarkable immunosuppressive properties [155, 156, 186]. Nowadays, MSCs have been derived from many different organs and tissues, but much of the literature is on MSCs from bone marrow or adipose tissue [187]. Recently developed a general, minimal working definition of MSC isolates that rests on three criteria: plastic adherence at *in vitro* culture; molecular surface profile (expression of CD105, CD73, and CD90 but lack of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and human leukocyte antigen DR); and *in vitro* differentiation into osteoblasts, chondrocytes, and adipocytes. These criteria apply to the human placenta-derived MSCs and human adipose tissue-derived



MSCs, which were used in the present study [188]. Interactions between MSCs and PEM substrates have not been studied before.

## 5.2. Interactions between MSCs and native (PLL/HA) substrates

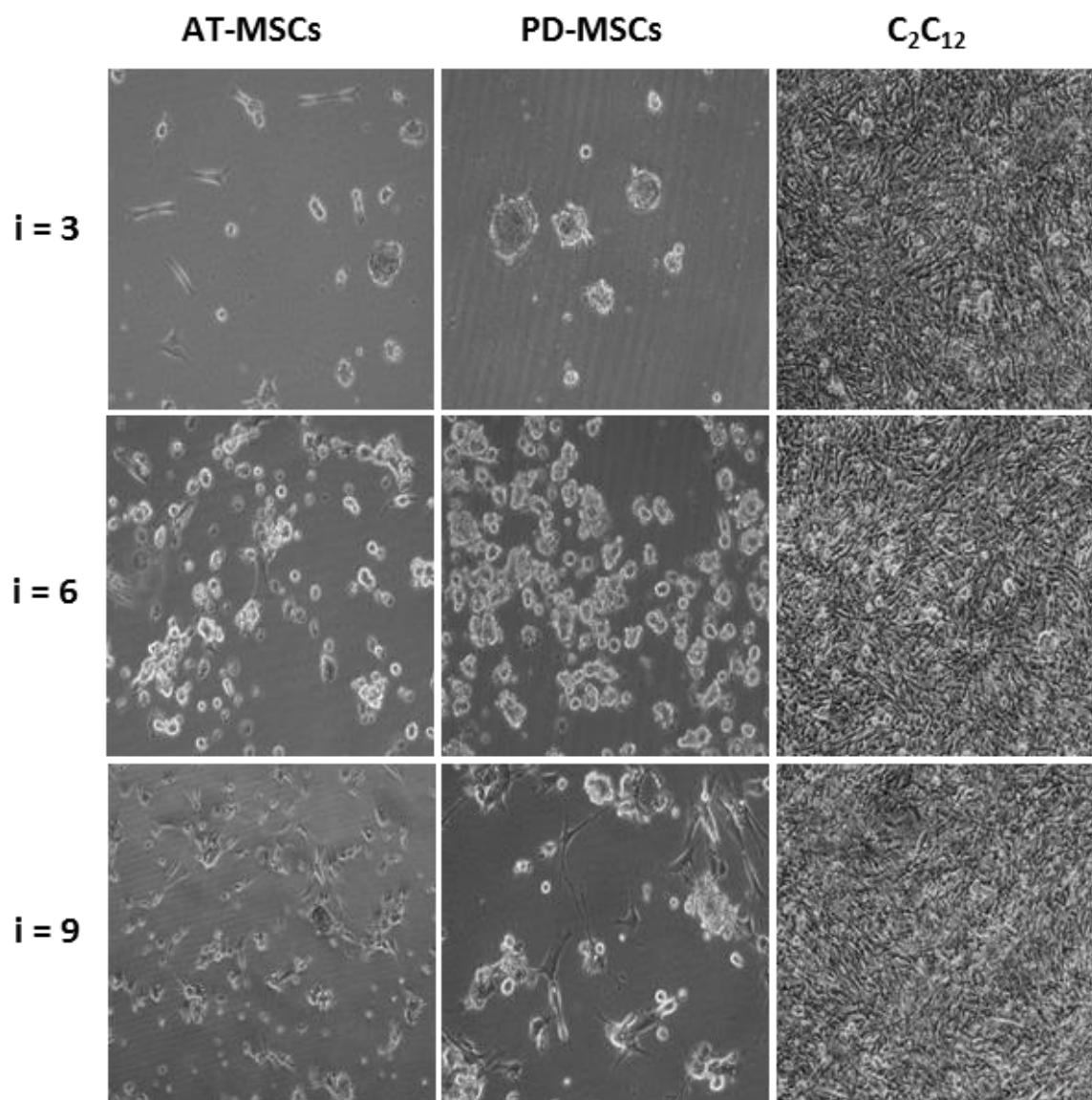
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To promote interactions with MSCs, the following parameters of PLL/HA multilayer build-up were controlled. (I) Thickness was varied according to the number of PLL/HA layer pairs. (II) Cell adhesiveness was approached through deposition of cell adhesion glycoprotein FN as the outermost layer. (III) Stiffness of the multilayer was increased by introducing covalent cross-links and amide bonds between HA and PLL. (IV) FN was covalently affixed or physically adsorbed onto multilayer. Coatings were prepared with three-, six-, or nine-layer pairs of (PLL/HA), followed by an end layer of positively charged PLL and the final (optional) adsorption of FN. The resulting constructs were designated (PLL/HA)<sub>i</sub>-PLL-FN, where *i* is the number of layer pairs. The stepwise adsorption of PLL, HA, and FN as monitored *in situ* by OWLS as the example of a (PLL/HA)<sub>9</sub> coating is depicted on the **[Figure 32A]**. OWLS showed successful adsorption of FN, which assumed surface densities between 350 and 700 ng/cm<sup>2</sup> on three-, six, or nine-layer-pair constructs **[Table 4]**. These adsorbed FN surface densities are biologically significant. Saturation levels from 350 to 400 ng/cm<sup>2</sup> were previously reported to represent approximately the amount of FN necessary to produce a monolayer coating based on the dimensions of the molecule [189, 190]. Such densities are known to support strong cell adhesion on solid synthetic surfaces as well as on thin multilayer films [191]. None of the FN-terminated (PLL/HA) multilayer substrates, when used as built, functioned as adhesive substrate for PD-MSCs or AT-MSCs **[Figure 32 B]**. Both types of MSCs seeded at density  $3.5 \times 10^4$  cells/cm<sup>2</sup> attached poorly. This was not from the toxicity of the PEM compounds, because cells could adhere and spread at the bottom of the same well on the tissue culture plastic near the coated glass slide (not shown). It was also not from loss of function of FN from its adsorption to PEM substrate. We tested the mouse myoblast cell line C2C12, which was previously used to examine effects of substrate-dependent changes in FN conformation for integrin ligation and cell adhesion on synthetic surfaces [189]. All native (PLL/HA)<sub>3,6,9</sub>-PLL-FN multilayers were adhesive for C2C12 cells, and under the high seeding density

**A**



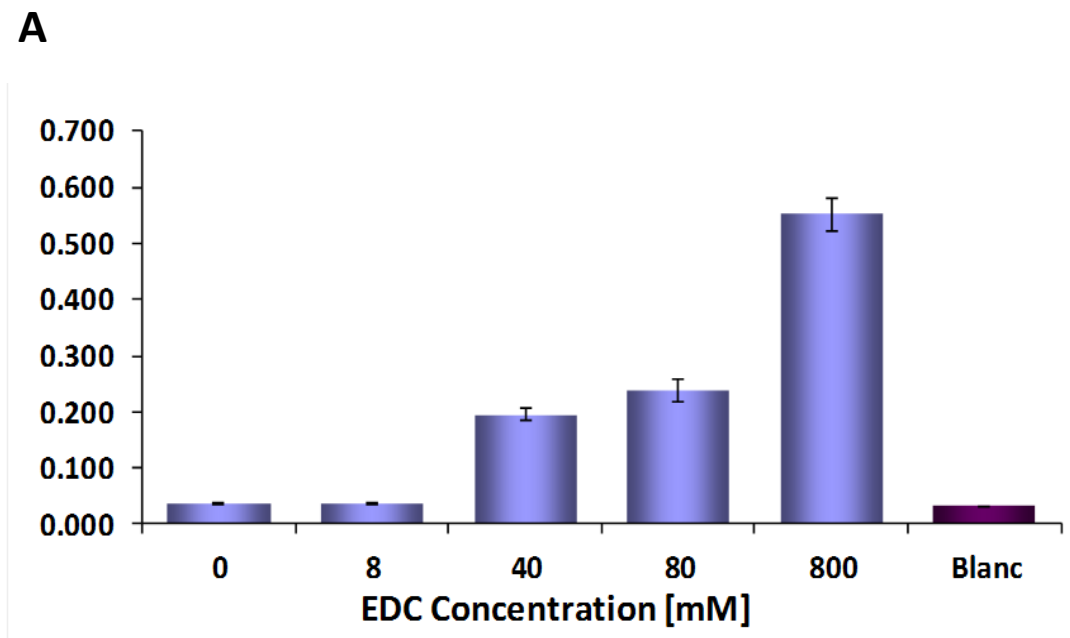
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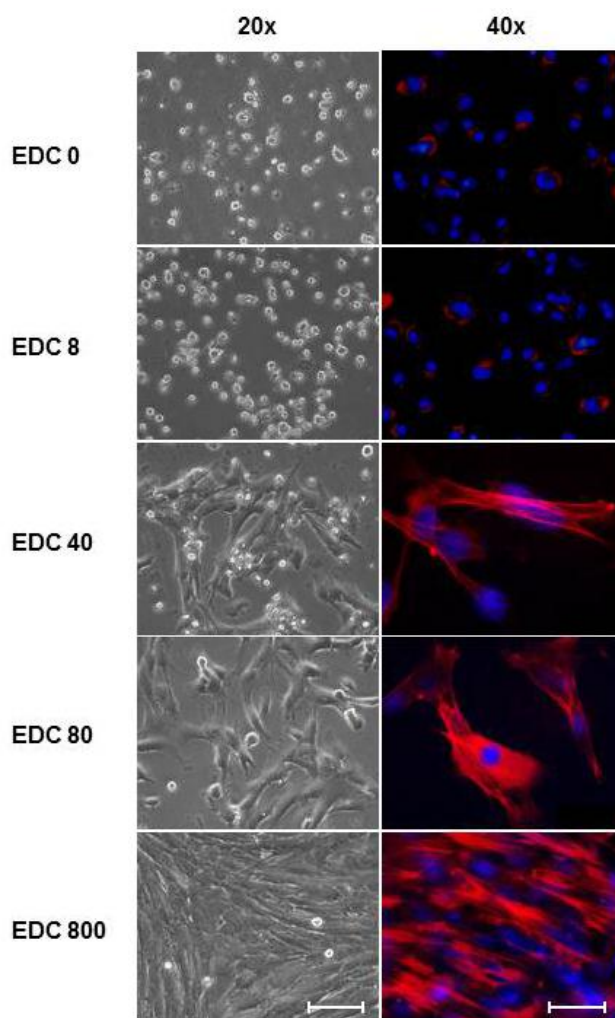
**Figure 32: MSCs do not adhere to native cationic poly-L-lysine and anionic hyaluronic acid (PLL/HA) multilayer coatings. (A) Representative optical waveguide light mode spectroscopy (OWLS) measurement of PLL/HA multilayer build-up using the example of a nine-layer pair coating. Coatings were prepared with a PLL end layer and terminated with adsorbed fibronectin (FN). Subsequent cross-linking was also followed *in situ* using OWLS. (B) Phase micrographs of MSC cultures on (PLL/HA)*i*-PLL-FN multilayer coatings 48 h after seeding, where *i* denotes the number of PLL/HA layer pairs. Bar size: 100  $\mu$ m**

### 5.3. Interactions between MSCs and cross-linked (PLL/HA) substrates

Recent studies of (PLL/HA) multilayer surfaces have revealed that stiffening of bulk multilayers enhanced adhesion of smooth muscle cells, chondrosarcomic cells, or osteoblasts [139, 181, 192]. Protocols have been described for controlled stiffening of (PLL/HA) bulk multilayers by converting the purely electrostatic cross-linking of the native multilayers (ionic interactions) to covalent cross-linking between ammonium groups of PLL with carboxylate groups of HA (amide bonds) using EDC in combination with sulfo-NHS [182, 193]. We tested (PLL/HA)<sub>9</sub>-PLL-FN multilayers cross-linked to different degrees by varying concentration of EDC from 0 to 800 mM in the EDC/NHS cross-linking mix for their ability to mediate PD-MSC adhesion [**Figure 33**]. Phase microscopy 72 h after cell seeding showed that a gradual increase of multilayer stiffness correlated with a gradual increase of well-spread, elongated PD-MSCs with well-organized actin fibres. MSCs on native (EDC 0) or low-cross-linked (PLL/HA) multilayers (EDC 8) remained roundish and loosely attached to the multilayers; they lacked actin fibres or displayed cortical actin organization. Visual inspection indicated that multilayer coatings formed under the highest cross-linking regime, EDC 800, were optimal for MSC adhesion.



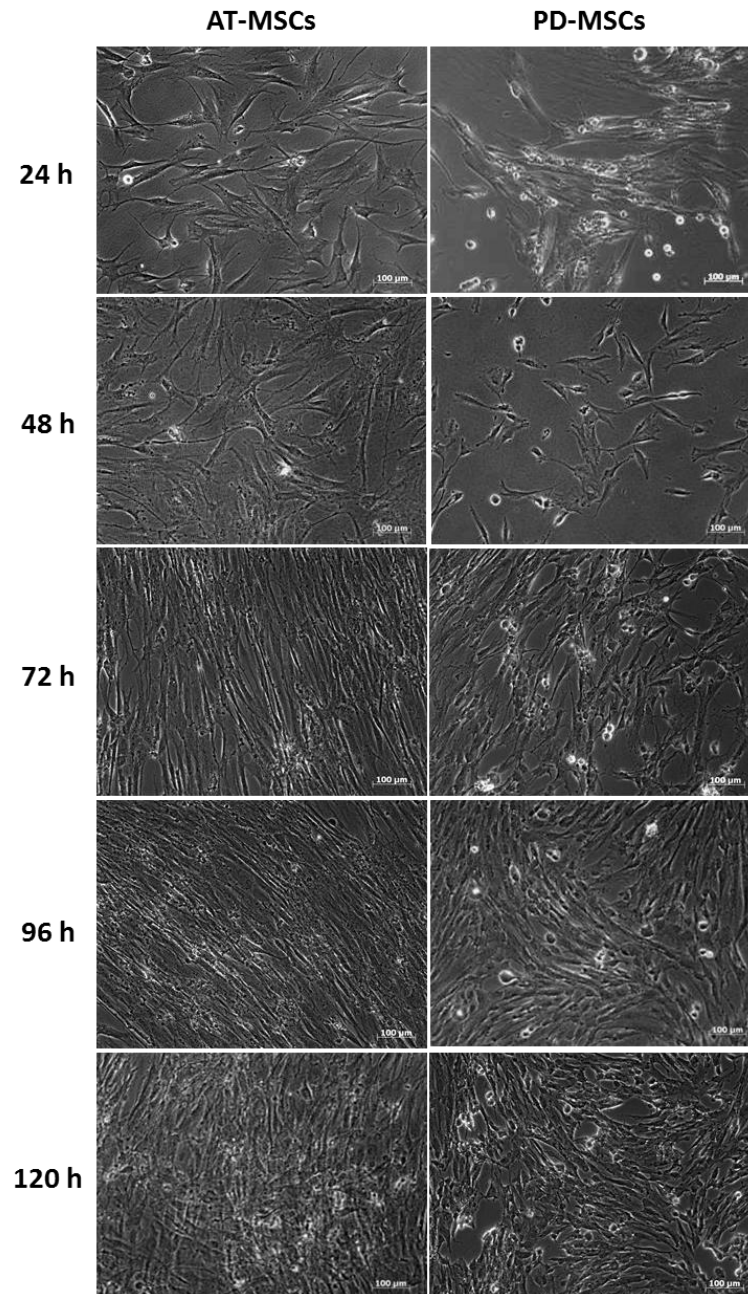
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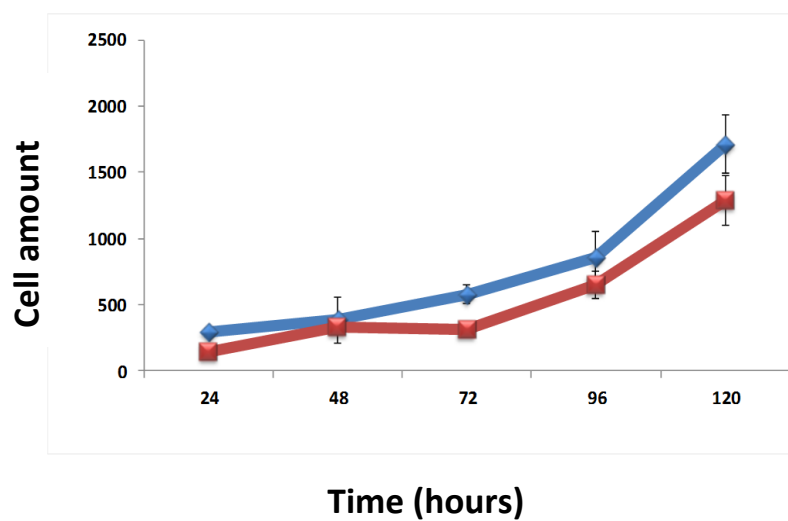


**Figure 33: Stratification of stiffened multilayers for promotion of MSC adhesion. (A) Determination of cell densities on native multilayers or multilayers cross-linked with EDC. (B) Images show PD-MSCs on PLL/HA multilayers that were stiffened to various degrees by crosslinking with increasing concentrations of N-(3 dimethylaminopropyl-N)-ethylcarbodiimide hydrochloride (EDC) in combination with a fixed concentration of 100mM N-hydroxysulfosuccinimide sodium salt. The EDC numbers indicate the millimolar concentrations of EDC in the cross-linking mix. Images were taken 72 h after cell seeding. Phase contrast microscopy of cell morphology and fluorescence microscopy of actin cytoskeleton (red colour) show that stiffer coating correlated with greater cell anchoring and spreading. Cell nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; blue colour). Bar size: 50  $\mu$ m.**

Phase microscopic follow-up of low-density MSC cultures on cross-linked multilayers showed that MSCs maintained their ability to proliferate **[Figure 34 A]**. Cell counts obtained from DAPI-stained cultures revealed that PD-MSCs and AT-MSCs exhibited similar proliferation rates **[Figure 34 B]**.

**A**



**B**

**Figure 34: Proliferation of MSCs on cross-linked multilayers. (A) Phase microscopic monitoring of MSC cultures on (PLL/HA)<sub>9</sub>-PLL-FN multilayers cross-linked with the EDC 800 regimen. Bar size: 100  $\mu$ m. (B) Proliferation rates of PD-MSCs (blue) and human adipose tissue-derived MSCs (AT-MSCs) (red) on multilayers. Cell counts were determined from fluorescence micrographs of DAPI-stained cultures with image analysis software.**

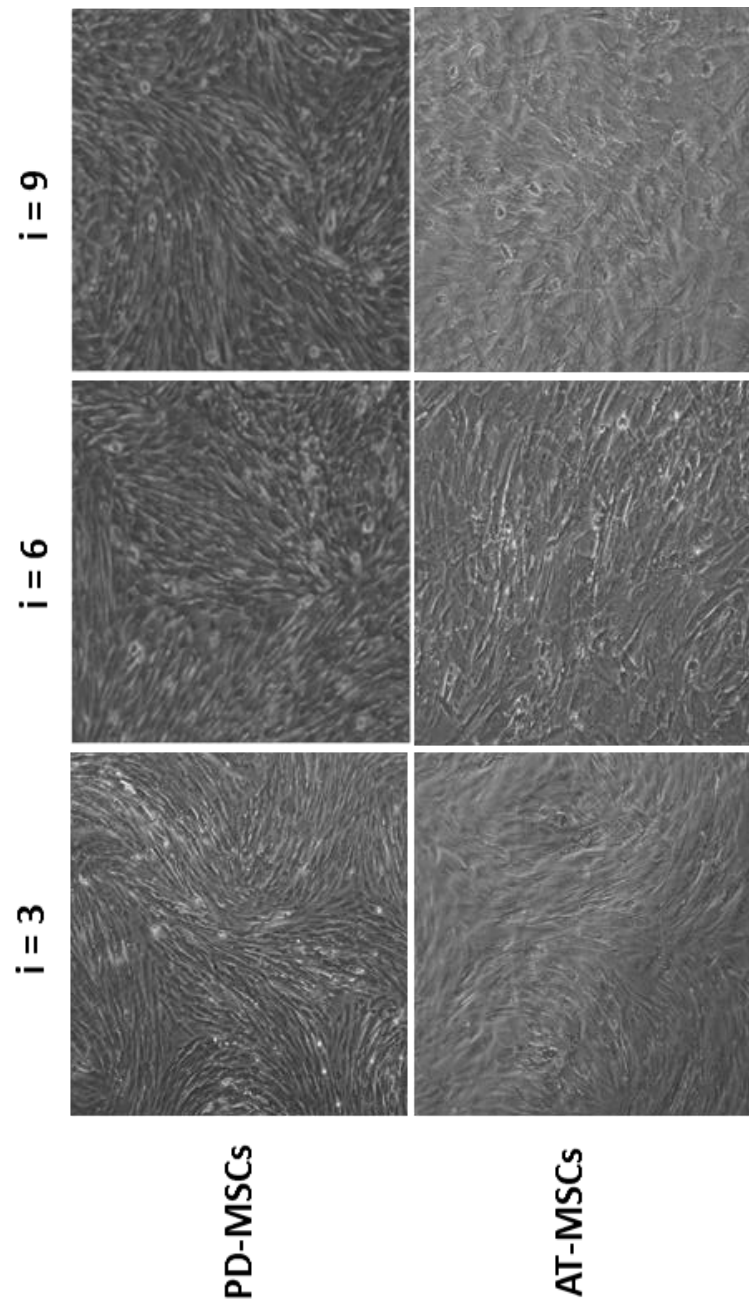
The stiffening of the bulk layer produced a strong positive effect for cell adhesion and outgrowth for multilayers of all thickness (i.e. (PLL/HA)-PLL-FN constructs with three-, six-, and nine-layer pairs) **[Figure 35]**. According to gross visual analysis, the positive effect of crosslinking for MSC adhesion appeared comparable between multilayers of different thicknesses. Cultures of MSCs seeded at density  $3.5 \times 10^4$  cells/cm<sup>2</sup>, formed dense PD-MSC and AT-MSC layers 48 h after seeding **[Figure 35 A]**. Quantitative analysis revealed that PD-MSC densities on multilayers from six and nine PLL/HA pairs were significantly higher ( $p < 0.05$ ) than those on three PLL/HA pairs **[Figure 35 B]**. Multilayer thicknesses did not affect AT-MSC densities. Without cross-linking, cell densities were low **[Figure 35 B]**. Summary of the results is shown in **[Table 4]**.

As next, I investigated the effect of cross-linking for the FN outer layer. For that, we compared the distribution of FN on cross-linked and native (PLL/HA)<sub>9</sub> layers, using fluorescently labelled FN, confocal microscopy, and data processing with Imaris imaging



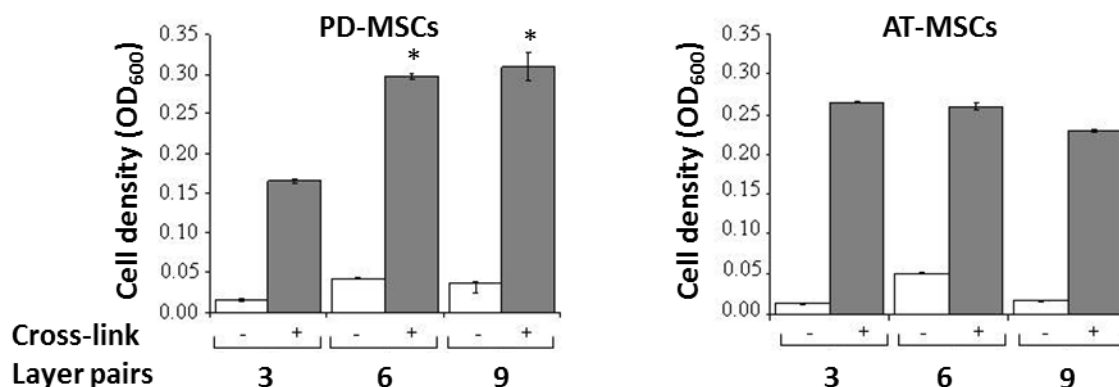
software. Three layer preparations with FN were compared: native bulk layer with physically pre-adsorbed FN, cross-linked bulk layer followed by physically pre-adsorbed FN, and cross-linked bulk layer with cross-linked FN [**Figure 36**].

**A**





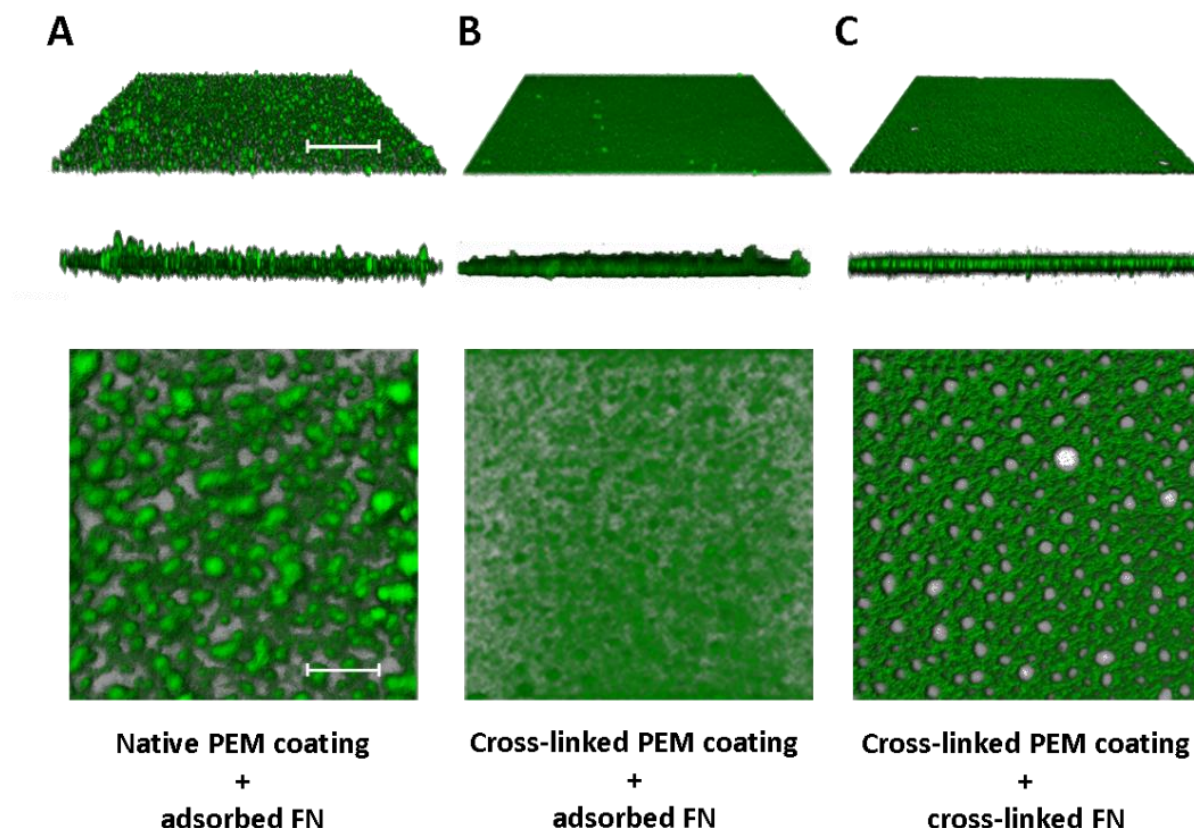
**B**



**Figure 35: Stratification of multilayer thickness for MSC adhesion. (A) Phase micrographs of MSC cultures. i denotes the number of PLL/HA layer pairs. According to gross visual examination with phase microscopy, there was no difference between cell densities on multilayers of different thickness. Bar size: 100  $\mu$ m. (B) Quantitative comparative analysis of cell densities on cross-linked and native PLL/HA multilayers, was performed using crystal violet staining and colorimetric analysis. In case of PD-MSCs, cell densities on cross-linked six- or nine-layered PLL/HA constructs were significantly greater than those on PLL/HA constructs with three layer pairs. In all constructs, the effect of cross-linking for greater cell density was highly significant compared with native ones. \*denotes  $p < 0.05$  versus native constructs; #denotes  $p < 0.05$  versus PD-MSCs on coatings with three layer pairs.**

THICKNESS		NATIVE PEM			CROSS-LINKED PEM	
(PLL/HA)i-PLL	FN (ng/cm) <sup>2</sup>	PD-MSC	AT-MSC	C <sub>2</sub> C <sub>12</sub>	PD-MSC	AT-MSC
i=3	350	poor	poor	complete	complete	complete
i=6	550	poor	poor	complete	complete	complete
i=9	750	poor	poor	complete	complete	complete

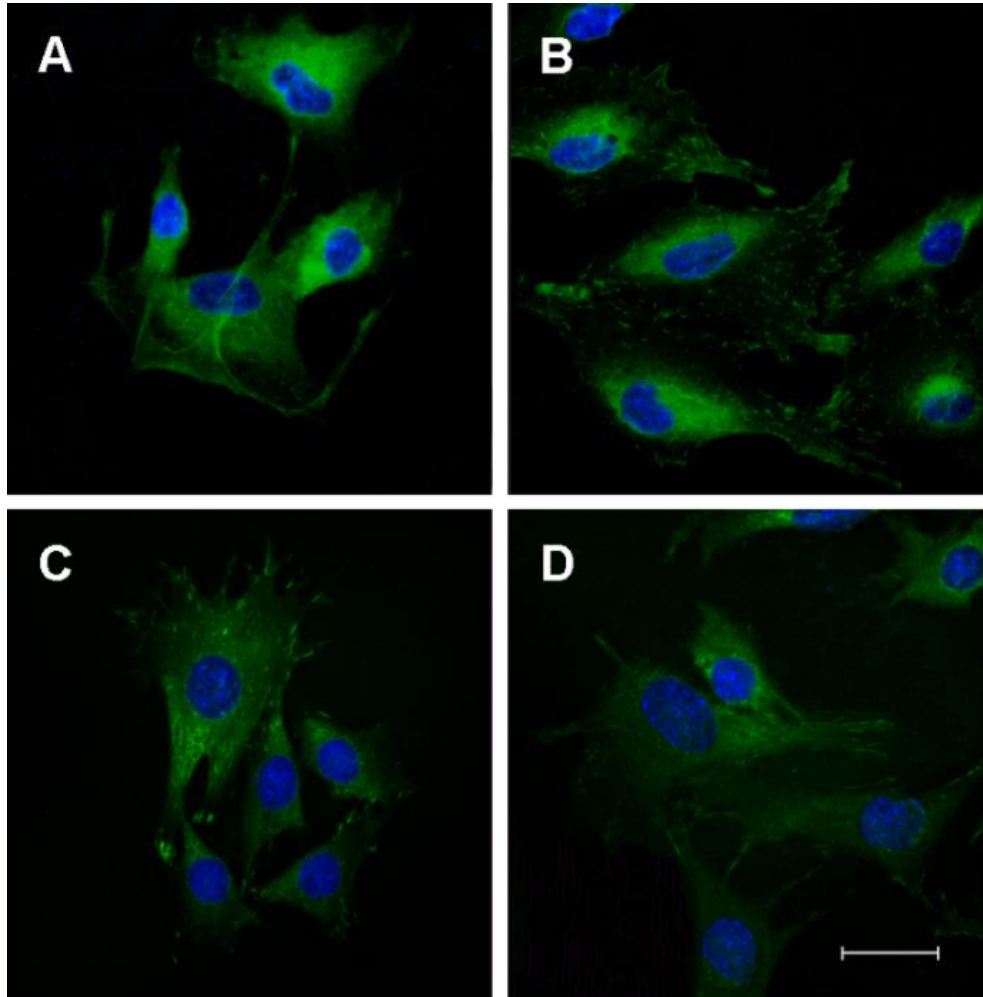
**Table 4: Adhesion of different cell types on (PLL/HA) coatings depending on the thickness of the coating. AT-MSCs – adipose tissue derived mesenchymal stem cells, PD-MSCs – placenta-derived mesenchymal stem cells, C2C12 – mouse myoblasts.**



**Figure 36: Cross-linking of PLL/HA layers affects FN surface distribution.** Confocal microscopy of native or cross-linked (PLL/HA)<sub>n</sub> multilayers formed with a physically adsorbed or chemically coupled FN outer layer. The green colour represents the fluorescently labelled FN. (A) Native PLL/HA multilayer with adsorbed FN. (B) Cross-linked PLL/HA multilayer with adsorbed FN. (C) Cross-linked PLL/HA with cross-linked FN. Top row, three-dimensional reconstitutions of FN top layers with Imaris software. Bar size: 30 mm. Middle row, side views of the FN interfaces. Bottom row, top views of the FN interfaces; the high magnification images were produced using a 7 x electronic zoom. Bar size: 4.3  $\mu\text{m}$ .

This analysis revealed different FN surface patterns. Top- and side-view images of illustrate the different FN interfaces [Figure 36]. Native and cross-linked PEM layers with physically adsorbed exhibited a diffuse, spotty, and spiky surface distribution of FN [Figure 36 A and B]; by contrast, chemically coupled FN appeared condensed in a narrow zone [Figure 36 C]. At the resolution of our confocal microscope, the chemically coupled FN appeared as fine regular mesh forming from coalescing droplets.

The condensed distribution of chemically coupled FN suggests greater FN surface density at the interface with cells. The stiffening of PEM layers by cross-linking along with concomitantly greater FN ligand density at the cell – (PLL/HA) coating interface had a profound effect on focal adhesion formation of MSCs, as demonstrated by immunofluorescence staining for vinculin, a component in the focal adhesion protein complex [Figure 37].



**Figure 37: Immunofluorescent staining for focal adhesion formation on native and cross-linked FN-terminated (PLL/HA)<sub>9</sub> layers. Cells were stained for focal adhesion protein vinculin (green) and cell nuclei counterstained with DAPI (blue). Confocal images of PD-MSCs on (A) native and (B) cross-linked PLL-HA layers. (C) C2C12 mouse myoblasts on native and (D) cross-linked PLL/HA layers. Bar size: 30  $\mu$ m.**

MSCs grown on cross-linked PEM layers, but not those on native PEM layers, showed punctuate expression of vinculin at the tips of elongated cell structures **[Figure 37 A, B]**.

These results indicate that the functionality of FN for binding to the FN adhesion receptor, integrin  $\alpha_5\beta_1$ , on cells was not affected. MSCs did not attach and spread well on cross-linked (PLL/HA) layers with physically adsorbed FN (data not shown). Parallel vinculin staining of the murine C2C12 myoblasts also showed focal adhesions on native layers and little effect of cross-linking the bulk layer and FN **[Figure 37 C, D]**.

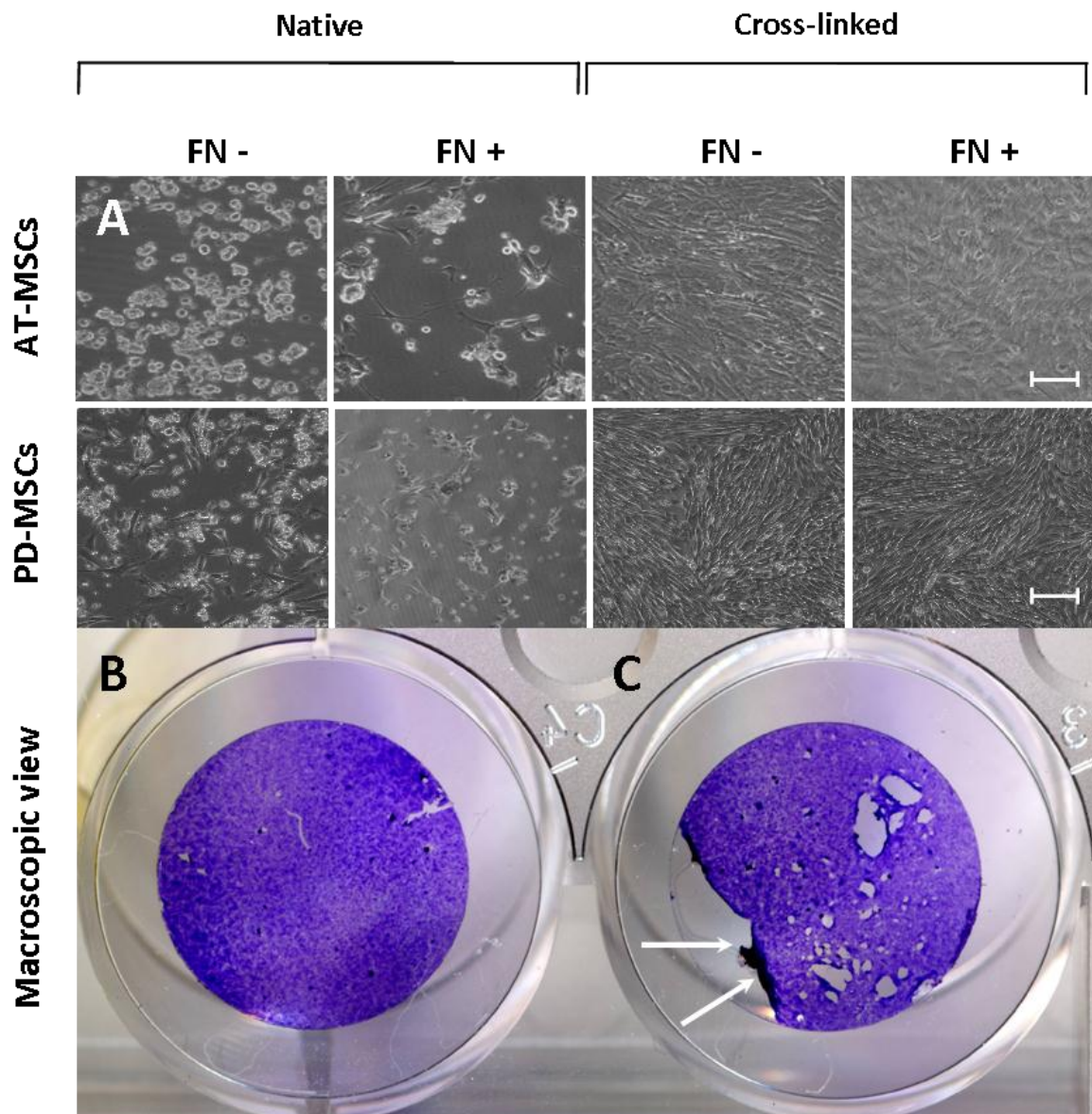
However, we noted that cell areas of C2C12 cells on native layers often appeared larger than on cross-linked layers, and cells exhibited many filopodia. In conclusion, cross-linked PLL/HA multilayer substrates formed with cross-linked FN possess better adhesion properties for MSCs.

Our next steps of investigation concerned long-term stability of densely grown MSC cultures and their differentiability on (PLL/HA) multilayers. We examined the role of the outer layer of FN for stability of dense MSC cultures, comparing side-by-side physically adsorbed FN with covalently affixed FN **[Figure 38]**.

Covalent binding of FN to multilayers was critical for maintenance of dense MSC cultures, whereas purely physical FN adsorption performed insufficiently **[Figure 38 A]**. Only MSC cultures on covalently cross-linked FN were complete after 1 week **[Figure 38 B]**. Beyond this period, the cultures gradually began to detach focally (holes in the cell layer), especially under the conditions of differentiation culture in osteogenic or chondrogenic medium; spontaneous lift of MSC layer as sheets rolling up from the edges was occasionally observed **[Figure 38 C]**.

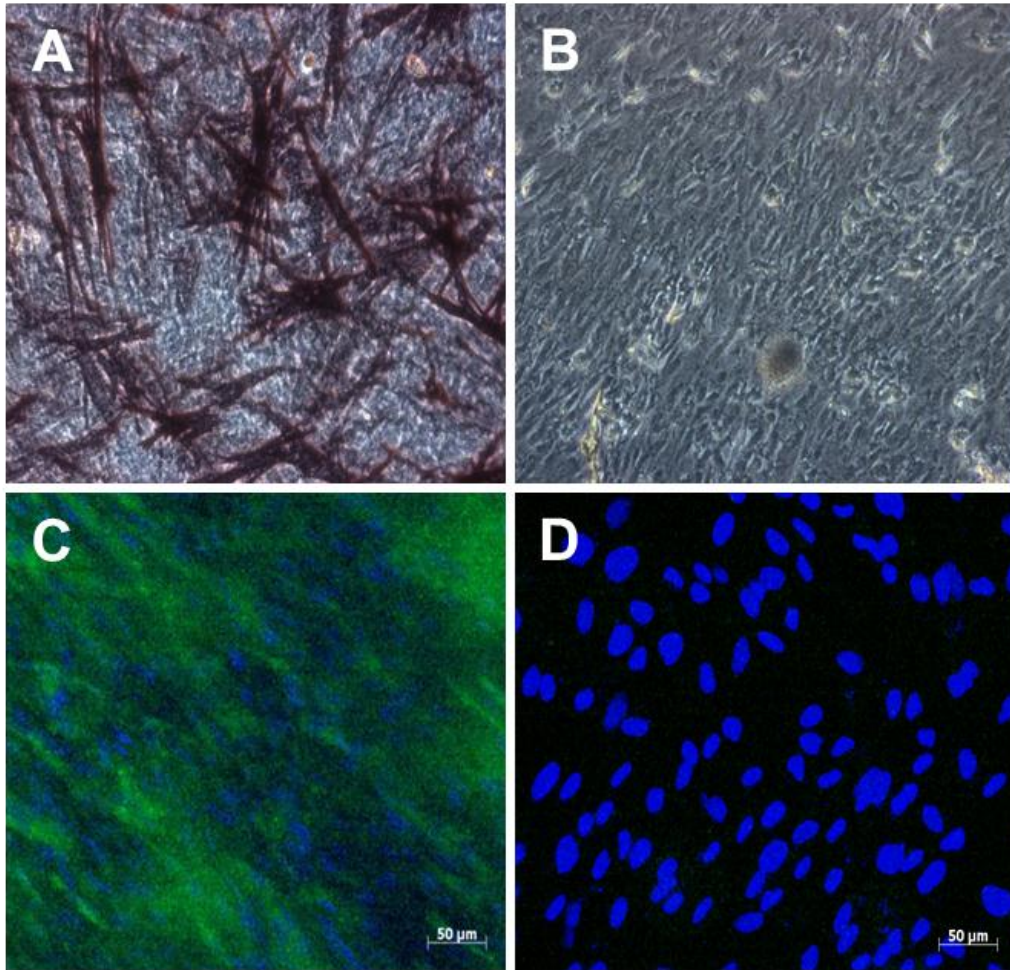
Cultures of PD-MSC and AT-MSC (data not shown) on cross-linked (PLL/HA)<sub>9</sub>-PLL-FN remained capable of differentiating into mesodermal lineages, as demonstrated by biochemical stainings for chondrogenic and osteogenic differentiation markers, respectively **[Figure 39]**.

Differentiation of PD-MSCs and AT-MSCs towards adipogenic lineage on cross-linked (PLL/HA)<sub>9</sub>-PLL-FN substrates was not successful.



**Figure 38: The role of terminal multilayer-bound FN for initial MSC monolayer formation.** (A) Native and cross-linked (PLL/HA)<sub>9</sub>-PLL multilayer constructs were prepared with or without FN and seeded with PD-MSCs and AT-MSCs. Phase micrographs of MSC cultures on multilayer 48 h after seeding. Stiffness, as accomplished using EDC=NHS crosslinking of multilayers, was critical. Pre-adsorption of FN showed unnecessary for initial adhesion and outgrowth of MSCs. (B, C) Effect of physical FN adsorption (B) versus chemical cross-linking of FN (C) to PLL/HA multilayers for maintenance of MSC layers. Images show crystal violet stained PD-MSCs monolayers after 1 week in culture. Covalent affixation of multilayer-bound FN proved important for preventing partial detachment.



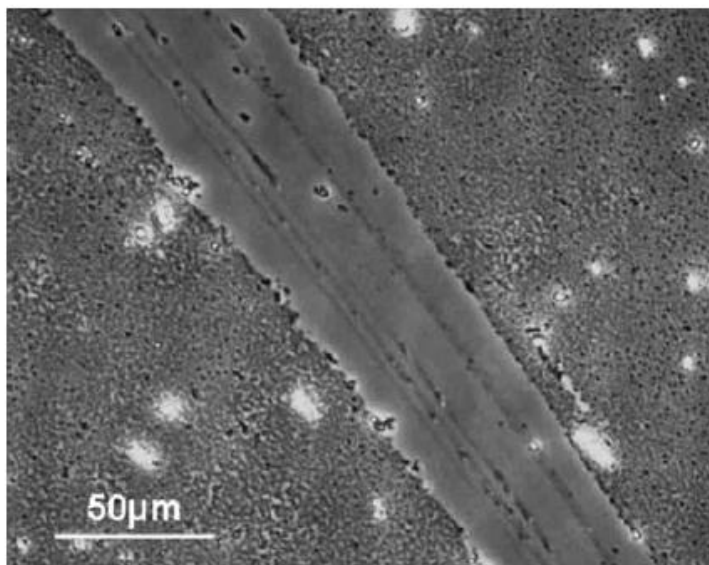


**Figure 39: Multilayer-bound MSCs remain capable of mesodermal lineage differentiation upon culture with soluble induction factors. Images show PD-MSC cultures on (PLL/HA)9-PLL multilayers with covalently affixed FN. (A) Induced osteogenic differentiation of PD-MSCs. The differentiated PD-MSCs were stained for ALP activity. (B) Control culture in standard medium showed no differentiation. (C) Induced chondrogenic differentiation was demonstrated by immunofluorescent staining with antibodies for collagen II (green). Cell nuclei were counterstained with DAPI (blue). (D) Control culture in standard medium showed no differentiation. Blue, DAPI stained nuclei. Bar size: 50  $\mu\text{m}$ .**

#### 5.4. Relevance of (PLL/HA) substrates for stem cell sheet engineering

For full PEM-driven control of cellular response, continuous PEM coating was sought to minimize influences from the underlying glass substrate on which the PEM coatings were built up. Deposition of eight-layer pairs of (PLL/HA) was previously found to be

sufficient to produce continuous PEM coating with a smooth surface, which was confirmed under our deposition conditions [132]. This can be readily appreciated from the image given showing a (PLL/HA)<sub>9</sub> coating with an experimental scratch to prove the coating [**Figure 40**].



**Figure 40:** Deposition of nine PLL/HA layer pairs produced continuous films. The coating was prepared on glass coverslips as described in the Methods section. An experimental scratch was created to demonstrate the continuous coating. The phase micrograph image was taken with a Leica DMIL fluorescence microscope equipped with a DML digital camera (Leica) at the 40x magnification.

Numbers (PLL/HA) layer pair did not influence MSC adhesion in the native or cross-linked condition, which suggests that the influence of the glass substrate on MSC adhesion response was minimal [**Figure 33**]. The mechanical properties, not the thickness, of (PLL/HA) multilayers were critical for MSC adhesion. As shown in earlier studies, post-formation chemical cross-linking of PEM layers with EDC/NHS limits polyelectrolyte diffusion inside the bulk PEM structure and increases its stiffness without significant change of PEM thickness [139]. Fibronectin-terminated (PLL/HA) multilayers processed with a high cross-linking regime promoted PD-MSC and AT-MSC adhesion instantly and efficiently, whereas native multilayers, which are soft, were

almost adhesion-resistant to MSCs [193]. Our findings add to the broad notion that adhesive spreading of cells correlate with the effective stiffness of (PLL/HA), as well as other PEM coatings, and more generally with the stiffness of synthetic material substrates. How MSCs probe and adapt to the stiffer PLL/HA substrate is beginning to be understood. Focal adhesions provide MSCs with the necessary force transmission pathways to “feel” their gel matrix microenvironment through actin-myosin contractions [194]. Stiffer substrates produce stiffer and increasingly tense cells [194]. Studies with murine NIH3T3 fibroblasts plated on FN-coated polyacrylamide gels showed that cells change their internal stiffness, governed by cytoskeletal assembly and production of internal stresses, to roughly match the stiffness of the substrate [195]. Some cell types seem capable of anchoring even to soft multilayer substrates. We found that mouse C2C12 myoblasts, unlike the MSCs, adhered and spread on soft, native (PLL/HA) multilayers [**Figure 32 B**] and formed vinculin-positive focal adhesions [**Figure 32 C, D**]. Reasons for different adhesion responses of MSCs and C2C12 cells on native multilayers with surface-adsorbed FN remain speculative. It may be the result of different “inside out” signalling pathways in C2C12 cells and MSCs that lead to different activation stages of the responsible FN adhesion receptor,  $\alpha 5 \beta 1$ . A two-stage activation mechanism for  $\alpha 5 \beta 1$  binding to surface adsorbed FN was described [196]. Another possibility is that adsorption of FN to a (PLL/HA) multilayer substrate produces a conformational change in FN and that this altered FN is bound by  $\alpha 5 \beta 1$  on C2C12 cells and MSCs at different levels. It has been shown that adsorption of FN to substrates changes FN conformation and subsequently alters its binding to the integrin  $\alpha 5 \beta 1$  [189]. Finally, C2C12 myoblasts may possess an inherent natural capacity to comply with soft substrate environments. In support of the latter, emerging knowledge suggests that cellular responses to substrate stiffness (e.g., for maximal spreading or differentiation) are cell-type dependent in that the effective range of substrate stiffness depends on the tissue type from which the cells were derived [197]. The reported value for elasticity of striated muscle tissue is approximately 8 to 17 kPa, 30 which is within the range of elasticity reported for native PLL/HA coatings [193, 198]. Hence, it is conceivable that C2C12 myoblasts by nature are better prepared to attach and spread on soft PEM substrates. Recent studies have shown that tissue-level matching substrate



stiffness strongly affects lineage specification and morphology of human MSCs [194]. A simple alteration of substrate compliance evoked specific MSC differentiation (e.g., soft polyacrylamide gel substrates that mimic brain were neurogenic, whereas stiff substrates that mimic collagenous bone were osteogenic) [198]. In light of this emerging knowledge, PLL/HA multilayer substrates may offer great opportunities for compliance-specific differentiation assays of MSCs, because the Young's modulus of PLL/HA multilayer coatings can be readily modulated over a range from 0 to 400 kPa, depending on their cross-linking degree [193]. Along these lines, it has been reported that modulation of stiffness of PLL/HA multilayer substrates in turn modulated C2C12 myoblast cell differentiation and their organization into myotubes [199]. I investigated the role of FN outer layers for MSC culture initiation, focal adhesion formation, and maintenance of densely grown MSC cultures. PLL-terminated multilayers were chosen for study, because other studies suggested that positive-terminal PEM layers favourably influence the distribution of FN molecules by inhibiting their aggregation in the adsorption layer, unlike negative-terminal layers [200]. With regard to adsorbed FN mass, our OWLS measurements revealed that the negatively charged FN adsorbed to terminal layers of HA at a similar level, despite the negative charge and hydrated nature of the latter (data not shown). Confocal microscopic analysis of the FN interface revealed that crosslinking of FN-terminated (PLL/HA) layers changed the FN distribution from diffuse to condensed, resulting in greater presentation of FN adhesion sites to cells. Our results showed that the details for preparation of the FN interface on (PLL/HA) coatings matter greatly, at least for MSC culture. Only bulk layers with cross-linked FN were effective substrates for MSCs, which indicates that cross-linking does not affect the function of FN. It is likely that the stiffening by cross-linking plus the greater FN ligand density at the cell – PEM layer interface resulting from cross-linking synergistically accounts for the enhanced MSC spreading and focal adhesion formation **[Figures 36 and 37]**. Physically adsorbed and chemically coupled FN was found distributed only at the top of the (PLL/HA) coating. This confirms other studies of FN adsorption that have indicated that FN rapidly complexes with PLL with little or no FN penetration into the PEM bulk [191]. Covalent fixation of FN to (PLL/HA) coatings proved necessary to maintain densely grown MSC cultures during differentiation culture

over weeks **[Figure 38 B]**. The amounts of FN adsorbed to (PLL/HA) multilayers, as determined according to OWLS, were 350 to 700 ng/cm<sup>2</sup> **[Table 4]**. These amounts favourably compared the (high) plateau densities of adsorbed FN measured on bacterial or tissue culture polystyrene [189]. FN is known as flexible glycoprotein with substrate-sensitive conformations, which in turn can modulate cell proliferation and differentiation [189]. Our data indicate that EDC/NHS-mediated cross-linking of multilayer-bound FN does not impair its functionality. Similar observations were made for collagen coatings cross-linked with EDC/NHS, which led to greater endothelial cell adhesion and proliferation on vascular grafts than with physically adsorbed collagen [201]. Innovative use of charge-responsive polyelectrolyte coatings could provide a new means for non-enzymatic harvest of MSCs and their transplantation as intact, cohesive cell sheets into diseased tissues, a concept known as cell sheet engineering [126]. It has been already demonstrated that monolayered polycationic copolymer poly(l-lysine)-grafted-poly(ethylene glycol) that were formed on conducting metal oxide substrate desorbed from their metal oxide substrate after the application of a positive potential, lifting complete sheets of osteoblasts cultured on the polyelectrolyte monolayer [138]. Based on these results I proposed to use (PLL/HA) platform as Innovative charge-responsive polyelectrolyte coating for non-enzymatic charge-mediated harvest of PD-MSCs sheets. Surprisingly, my results show that native as-built (PLL/HA) coatings are very poor adhesive for PD-MSCs and only the cross-linked, FN-terminated (PLL/HA) coatings under present study permitted formation of complete MSC layers [188]. Unfortunately, my following experiments have shown that such coatings do not dissolve upon electrochemical polarization (unpublished observations). Thus, establishment of new alternative multilayer system that might be sufficient for formation and electrochemically controlled lift of MSC sheets was needed.

## 5.5. Achievements of the fifth chapter

In the second part of my thesis I established tuneable, polyelectrolyte multilayer substrates with biological features and physico-chemical properties that permit adhesion, proliferation, and differentiation of human MSCs. I was aiming to evaluate usability and functional potential of such type of substrates as a “smart” responsive,

biomimetic platform for engineering, characterization, and following non-invasive detachment of PD-MSC sheets, by application of electrochemical means [138]. Although the developed platform was shown to be not suitable for electrochemical harvesting of PD-MSCs this development was still essential to realize that the mechanical properties of the polymer substrate affect the cell-surface interactions and cell viability. The results of the next chapter therefore are a direct consequence of the findings here. In addition, it is quite likely that the cross-linked multilayers of this chapter could also be used for the electrochemically induced pH change based harvesting of cell sheets.

## CHAPTER 6

# RECOVERY OF PLACENTAL STEM CELL SHEETS

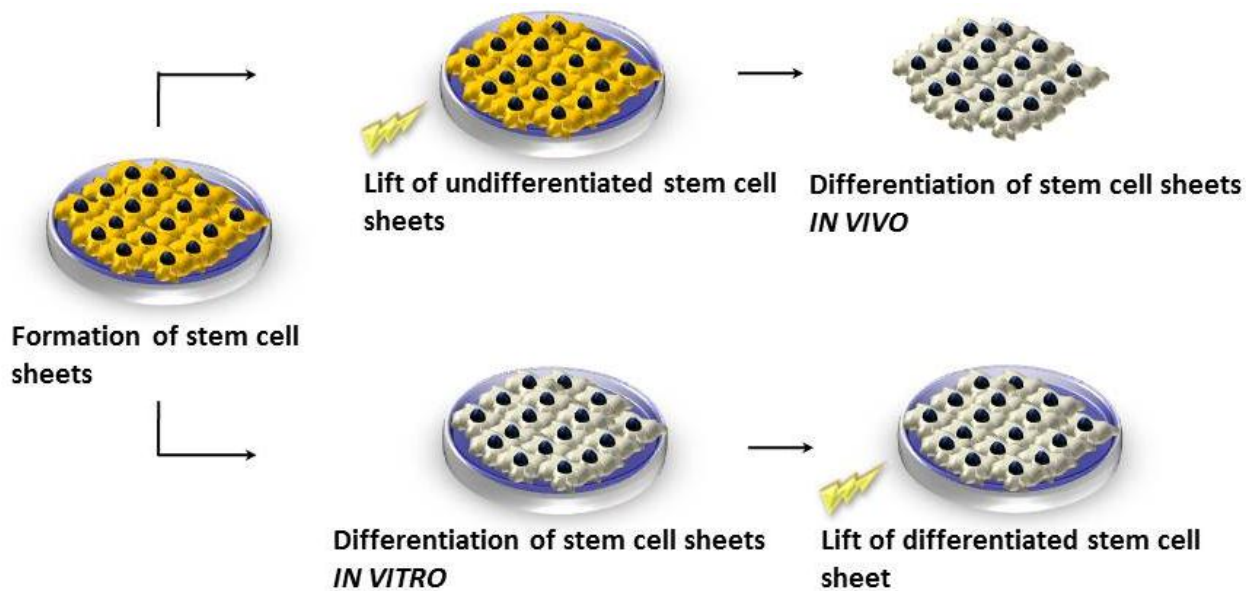
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*This chapter of the thesis is published as per-reviewed article entitled as “pH-controlled recovery of placental mesenchymal stem cell sheets” (BIOMATERIALS 2011, 32, 4376-4384). Atomic force microscopy analysis, as well as procedure of electrochemical peeling was performed by Dr. Orane Guillaume – Gentil. The process of stem cell sheet recovery has been microscopically acquired with assistance of Dr. Sara Andrea Schaufelberger and Philipp Lieneman.*

### 6.1. Biomedical applications of stiff (PAH/PSS) multilayer substrates

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Cell sheet engineering has been originally developed using cell culture dishes grafted with temperature responsive polymers, showing cell adhesive properties at 37°C, while switching to non-fouling properties below 32°C [202]. Later, alternative approaches for the release of cell sheets have been proposed, based on enzymatic, electrochemical or magnetic triggers [80, 203]. Among those, the electrochemical stimuli are particularly attractive due to their spatial and temporal precision, rapidity and reversibility, and the possibilities for automation and remote control [Figure 41].



**Figure 41: Cell sheet detachment using electrochemical stimuli.**

Previous platforms designed for cell sheet release under electrochemical control are based on the controlled dissolution of sacrificial substrates made of self-assembled monolayers (SAMs) or polyelectrolyte multilayers (PEMs) [138, 204, 205].

These platforms offer versatile, biocompatible substrates, simple and inexpensive processing, precise control, and are applicable to any surface geometries. PEMs in particular represent highly promising material for the bio-interface, providing a precise control over their chemical, physical, and mechanical properties, and the possibility to incorporate bioactive moieties such as drugs, nucleic acids, peptides, proteins or growth factors [206].

PEM coatings composed of poly-(allylamine hydrochloride) (PAH) and poly-(4-styrene sulfonate) (PSS), two synthetic polyelectrolytes, recently emerged as excellent cell substrates. *In vitro*, (PAH/PSS) substrates showed good cell adhesive properties and improved cell proliferation for a variety of cell types including endothelial cells [207, 208], fibroblasts [209, 210], osteoblastic cells [211], and hepatocytes [212], promoted adhesion and differentiation of endothelial progenitor cells derived from rabbit peripheral blood [213] or human umbilical cord-blood [214], and improved cell growth and cell viability of bone marrow-derived human mesenchymal stem cells compared to fibronectin-modified surfaces [215].

The incorporation of a growth factor or a chemo-repulsive protein into PSS-ending multilayers further allowed for the modulation of the growth and survival of neurons [216], whereas VEGF embedded in PAH/PSS assemblies enabled the stimulation endothelial cells proliferation [217]. While all these studies revealed their promising properties in cell studies, (PAH/PSS) coatings were recently evaluated *in vivo*. In a rabbit model, human umbilical arteries treated with (PAH/PSS) multilayers showed high graft potency and no inflammation 3 months after implantation, holding great promises for small vessel replacement [140].

When subjected to an electrochemical trigger, (PAH/PSS) were shown to be relatively stable, with thin films dissolving only slowly after a prolonged electrochemical stimulation. On the other hand the pH lowering, which results from electrochemically-induced water electrolysis at the electrode surface, was shown to extend a few hundreds of nanometre above the thin (PAH/PSS) films [218].

The cells cultured on a (PAH/PSS) substrates most probably interact with a layer of proteins adsorbed on the polyelectrolyte films from the growth medium or produced by the cells. The protein-substrate, protein-protein, and individual protein intra-molecular interactions result from the sum of multiple interactions of different nature (ionic, hydrophobic, hydrogen bonding, van der Waals, etc.), and subtle changes in the proteins microenvironment, such as a decrease in pH, are expected to induce significant changes in the adsorbed protein layer [219].

We therefore speculated that a local, electrochemically-induced pH lowering at the bio-interface could instigate cell sheet detachment, through perturbation of the protein layer on which cells adhere. In third part of my thesis a novel (PAH/PSS) based platform for cell sheet engineering is demonstrated, where a local or global pH lowering allowed for controlled detachment of PD-MSC sheets. The stability of (PAH/PSS) substrates against pH lowering, and their suitability for the culture of PD-MSCs were examined *in vitro*. The compatibility of the harvesting process with regard to the preservation of stem cell viability, phenotype and differentiation potential was evaluated.

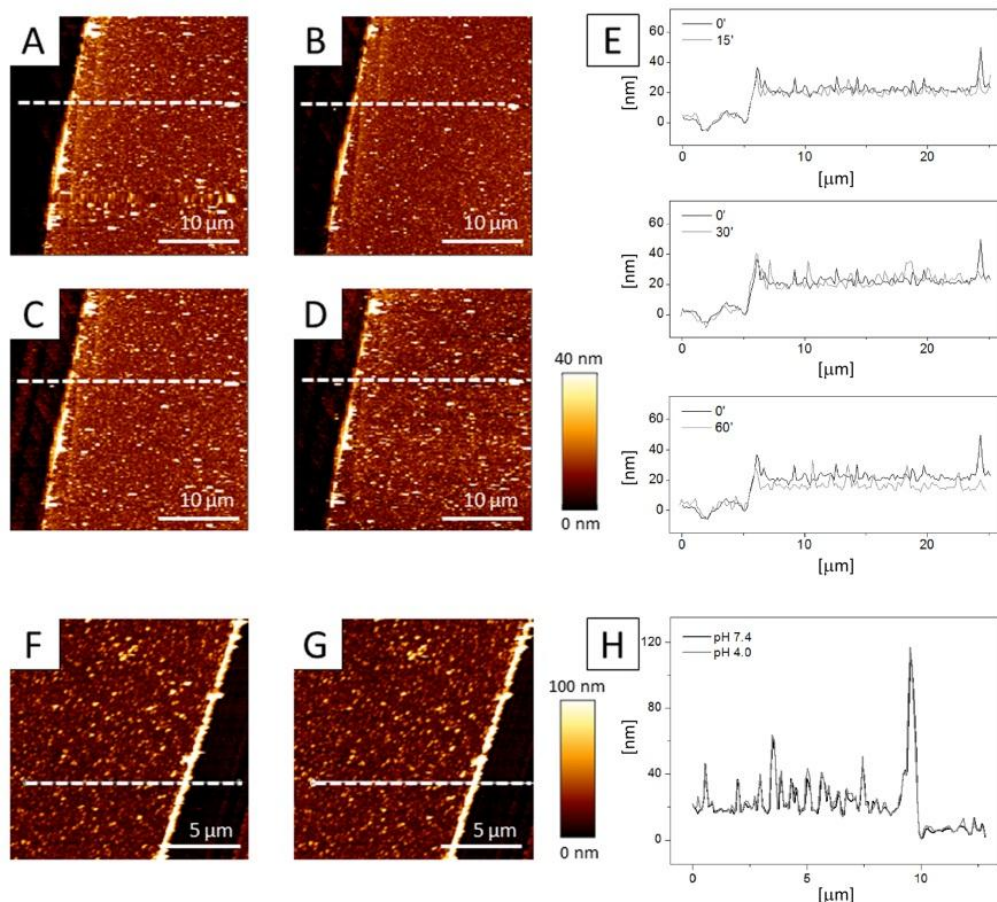
## 6.2. Characterization of (PAH/PSS) substrates

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Poly(allylamine hydrochloride) and poly(styrene sulfonate) (PAH/PSS) multilayered thin films were assembled layer-by-layer on indium tin oxide (ITO) electrodes to generate substrates for the growth and subsequent harvesting of placenta-derived mesenchymal stem cell (PD-MSC) sheets. The deposition of nine bilayers of (PAH/PSS) on the electrodes is yielded continuous thin films with a thickness of about 20 nm **[Figure 42 A and F]**.

The stability of the (PAH/PSS)<sub>9</sub> substrates in respect to an external electrochemical trigger was monitored by EcAFM. Under an applied current density of 30  $\mu\text{A}/\text{cm}^2$ , the (PAH/PSS)<sub>9</sub> films remained stable for at least 30 minutes, without any changes in the thickness or surface roughness (Data not shown). Applying a higher current density (100  $\mu\text{A}/\text{cm}^2$ ), no changes in thickness or surface roughness were detected by EcAFM measurements within 30 minutes, while a small decrease in thickness indicating the dissolution of the coating was detected after 60 minutes **[Figure 42 A-E]**.

The stability of (PAH/PSS)<sub>9</sub> films was further assessed towards lowering of the bulk pH. The coating, prepared under physiological conditions at pH 7.4, was immersed in a buffer at pH 4.0 for 2 hours. AFM images showed no changes in the coating thickness and surface roughness [Figure 42 F-H].



**Figure 42:** Effect of electrochemical stimuli and bulk pH decrease on (PAH/PSS)<sub>9</sub> thin films as followed by ecAFM. AFM Height mode images of a (PAH/PSS)<sub>9</sub> film deposited on an ITO electrode (A), and subsequently subjected to 100  $\mu\text{A}/\text{cm}^2$  for 15 (B), 30 (C) and 60 (D) minutes. The profiles (E), corresponding to the white dashed line in each AFM images, showed no significant changes at 15 and 30 minutes, while a slight decrease in thickness was observed after 60 minutes. (F) AFM height mode images of a (PAH/PSS)<sub>9</sub> film deposited on an ITO electrode in HEPES-2 buffer (pH 7.4) and (G) after 2 hours in a buffer at pH 4.0. The profiles corresponding to the white dashed lines on AFM images (H) showed no changes under the change of bulk pH. The black areas on each image correspond to scratches allowing for thickness determination.

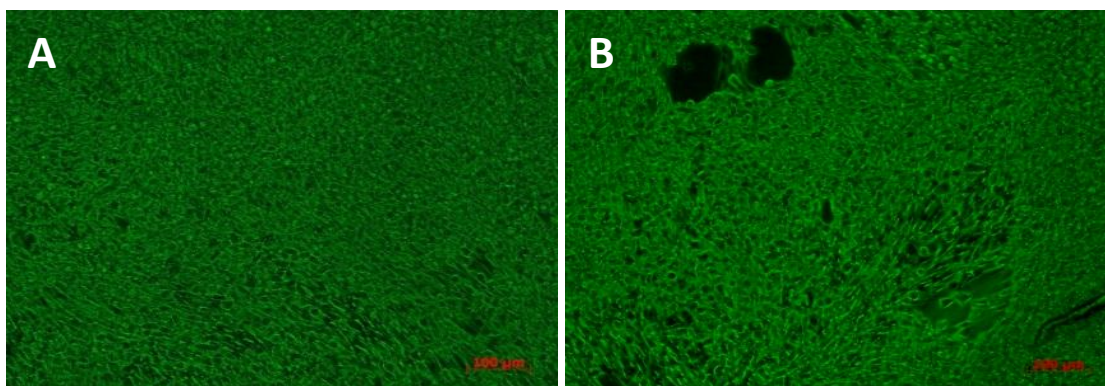


### 6.3. Assembling of PD-MSCs sheets on (PAH/PSS)<sub>9</sub> substrates

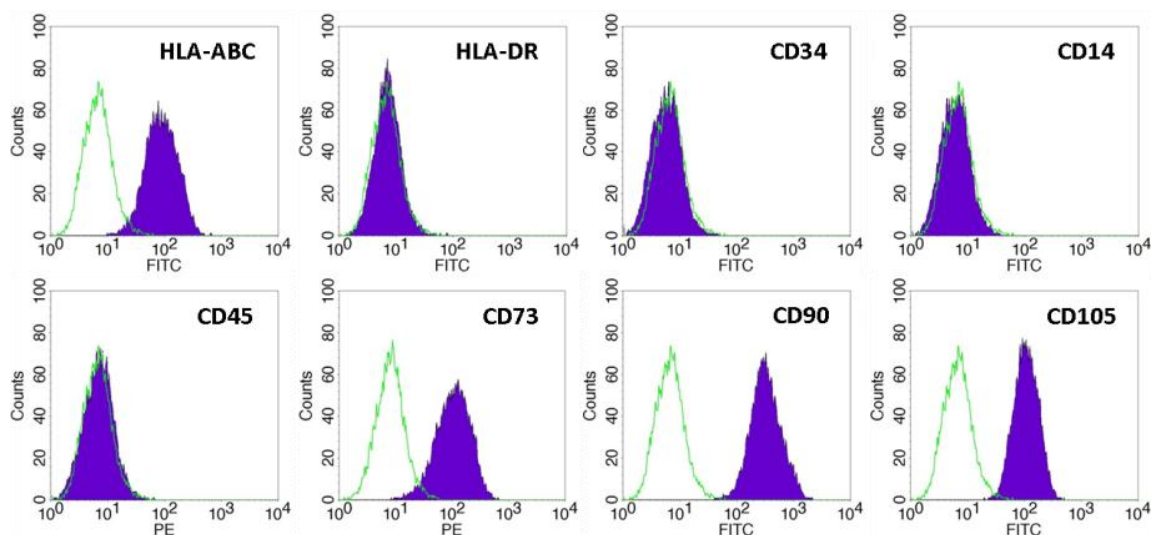
Placental MSCs (PD-MSCs) were cultured on the (PAH/PSS)<sub>9</sub>-modified electrodes to test the ability of the polyelectrolyte thin films to allow for adhesion and formation of confluent PD-MSCs layers.

The polyelectrolyte substrates induced normal cell anchorage, with the cells attaching easily and exhibiting fast proliferation. When seeded at high density ( $5 \times 10^5$  cells/cm<sup>2</sup>), PD-MSCs formed confluent monolayers within 4 hours. The viability and phenotypical stability of the PD-MSC sheets grown on the PAH/PSS substrates were determined by live-dead staining and FACS analysis, respectively.

Live-dead staining using calcein and ethidium homodimer showed that PD-MSCs in the confluent monolayers remained viable to a similar extent (95%) as cells grown in parallel under standard culture conditions on the TCPS plates (95%) **[Figure 43]**. Phenotypical characterization according the minimal criteria for determining of human MSCs stated by international society for cell transplantation (ISCT) indicated no significant changes in surface marker expression for the confluent PD-MSCs cultured on the PAH/PSS substrates [164]. Indeed, the cells were positive for HLA-ABC, CD73, CD90, and CD105 but negative for HLA-DR, CD14, CD34, and CD45 cell surface markers **[Figure 44]**.



**Figure 43: Viability of stem cell sheets before peeling. (A) Cell sheet generated from placental MSCs on the experimental (PAH/PSS)<sub>9</sub> coating; (B) Control cell sheet from placental MSCs grown on tissue culture polystyrene (TCPS).**



**Figure 44:** Phenotypic profile of engineered stem cell sheets before peeling. Flow cytometrical analysis (FACS) revealed that phenotypic profile of undifferentiated stem cell sheets fulfil the minimal criteria for defining multipotent mesenchymal stromal cells as stated by the international society for cellular therapy (ISCT). Green lines denote unstained negative control cells.

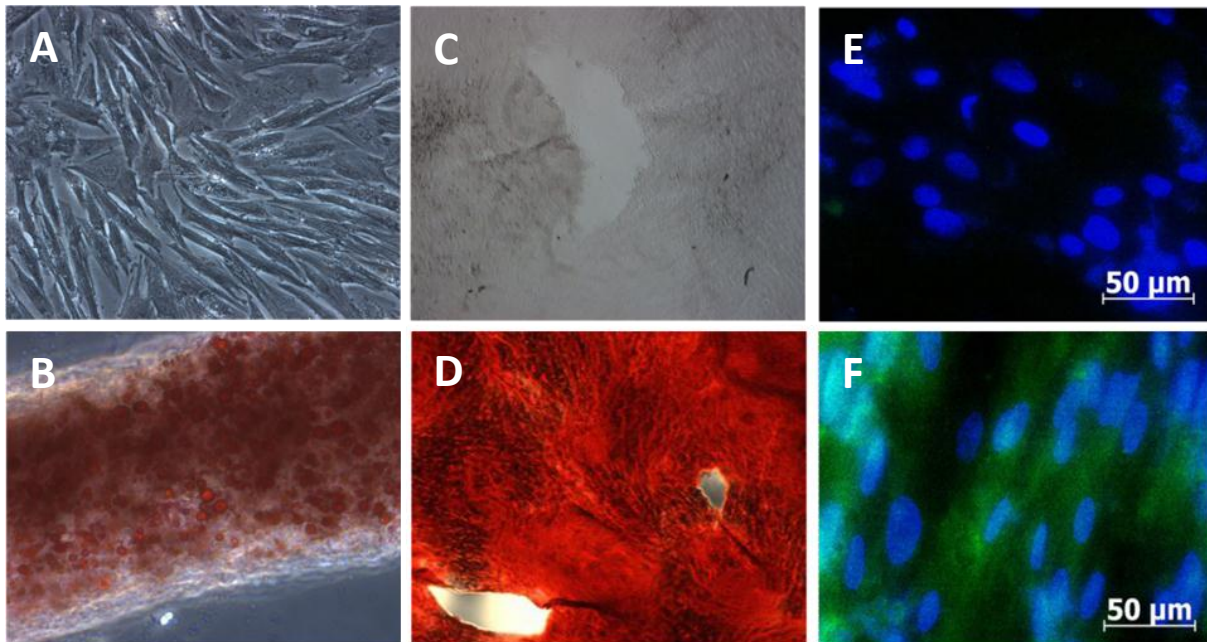
## 6.4. Plasticity of PD-MSC sheets grown on (PAH/PSS) substrates

### Medium-induced mesodermal differentiation of PD-MSC sheets

PD-MSCs cultured as dense cell monolayers were further tested to determine whether they retained their potential to differentiate *in vitro* towards the mesodermal lineage. Adipogenic, chondrogenic, and osteogenic differentiation were determined using specific biochemical and immunocytochemical staining [Figure 45].

Because typical mesodermal differentiation of MSCs takes three weeks, a stable adhesion of the cultured monolayers within this period was desired. PD-MSC monolayers remained stably attached to the (PAH/PSS)<sub>9</sub> coated ITO surfaces for the whole period of culture in control as well as in osteogenic, and chondrogenic differentiation conditions. Contrastingly, the PD-MSC monolayers upon adipogenic differentiation (21 days) starting to detach from the PEM substrates and rolled up [Figure 45 B]. PD-MSCs could be successfully differentiated towards mesodermal lineage. Deposition of big amount of lipid droplets, expression of alkaline phosphatase

as well as expression of collagen type II were determined for the cell sheets induced in adipogenic, osteogenic, or chondrogenic differentiation media respectively [**Figure 45 B, D and F**]. No sign of differentiation was observed for control, non-induced, PD-MSC monolayers [**Figure 45 A, C and E**].

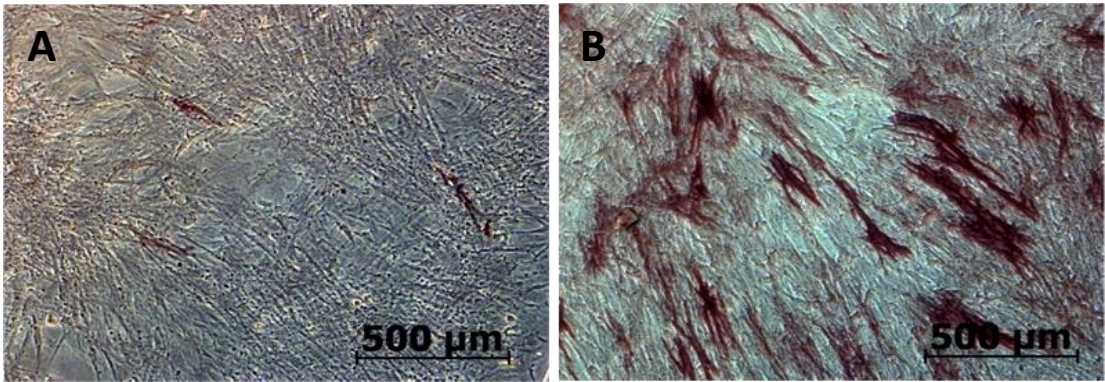


**Figure 45: Differentiation towards mesodermal lineage of PD-MSC monolayers cultured on (PAH/PSS)<sub>9</sub> coated ITO substrates. Control stem cell sheets cultured in standard conditions without induction do not show any sign of differentiation (A,C, and E). Adipogenic differentiation as demonstrated by Oil Red O staining of lipid droplets (B), Osteogenic differentiation as demonstrated by Alizarin Red S-staining of calcification (D), Chondrogenic differentiation as demonstrated by immunocytochemical Collagen Type II and DAPI staining (F).**

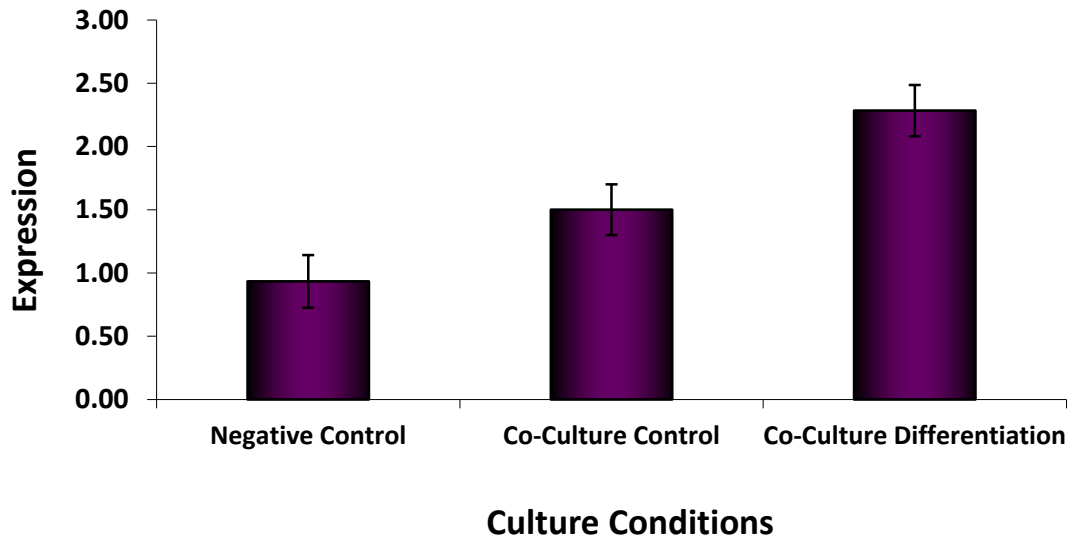
### **Osteogenic differentiation of PD-MSC sheets by indirect cell contact**

Instructed mesodermal plasticity has been additionally demonstrated by co-culture of PD-MSC sheets in indirect contact with human osteoblasts. Osteogenic differentiation was performed using standard co-culture system for organotypic cultures and determined by colorimetric analysis of alkaline phosphatase production and by analysis of collagen type I expression using real-time PCR.

Results obtained by colorimetric staining [Figure 46], as well as by real-time PCR [Figure 47] show clear difference in expression of alkaline phosphatase between samples in induced conditions comparable with control conditions (co-culture of PD-MSC with PD-MSC stimulated by OsteoDiff) as well as negative control conditions (co-culture of PD-MSC with PD-MSC without additional stimulation)



**Figure 46: Differentiation of PD-MSC sheets towards osteogenesis by co-culture. Alkaline phosphatase (ALP) expression in control conditions (A), and in induced condition (B) as determined by colorimetric analysis.**



**Figure 47: Differentiation of PD-MSC sheets towards osteogenesis by co-culture as determined by expression of human collagen type I using real-time PCR.**

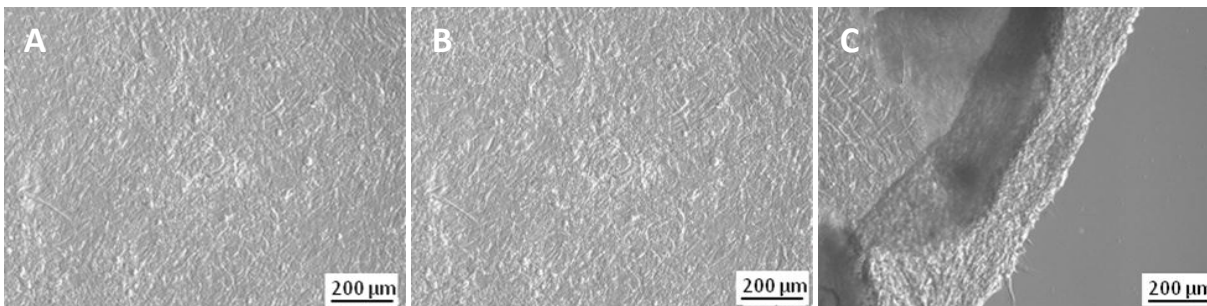


## 6.5. Harvesting of PD-MSCs sheets

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### Electrochemical trigger

Samples with confluent PD-MSC monolayers grown on (PAH/PSS)<sub>9</sub> coated electrodes were subjected to an electrochemical trigger to induce cell sheet detachment. While (PAH/PSS)<sub>9</sub> thin films were shown to be stable for at least 30 minutes under the same electrochemical conditions, the PD-MSC sheets completely detached from the substrate within 10 to 20 minutes when subjected to a current density of 30  $\mu\text{A}/\text{cm}^2$  [Figure 48].



**Figure 48:** Peeling of placenta derived mesenchymal stem cell (PD-MSC) sheets from conductive substrates upon electrochemical polarization. PD-MSCs were grown to confluence on ITO substrates coated with a (PAH/PSS)<sub>9</sub> thin film (A) and subsequently subjected to a current density of 30  $\mu\text{A}/\text{cm}^2$ . After 5 minutes of electrochemical treatment (B), the confluent cells remained attached to the substrate, while detachment of the whole, intact cell sheet was observed after 15 minutes (C).

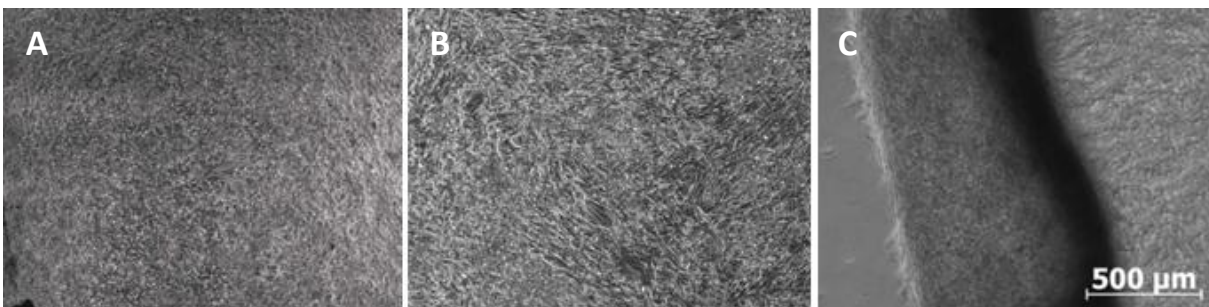
The PD-MSCs sheets were released from the substrate with intact cell-cell connections, and a fast adhesion was observed upon transfer to a new substrate, indicating the presence of extracellular matrix proteins under the harvested cell sheets.

### Bulk pH decrease

The effect of a decrease in environmental pH on the adhesion of the confluent PD-MSCs was further assessed. PD-MSC sheets grown at physiological pH (7.4) on (PAH/PSS)<sub>9</sub>-

coated ITO electrodes were exposed to cell culture media buffered at pH values ranging from 7.4 to 4.0.

The results demonstrated that cell attachment remained unchanged when the media was buffered above pH 5.0. However, when the pH value was reduced to 4.0, cells readily detached within 2-3 minutes as continuous, intact cell sheets **[Figure 49]**. The recovered cell sheets spontaneously attached when transferred on new dishes.



**Figure 49: Detachment of the PD-MSC sheets upon lowering of the bulk pH. Monolayer of PD-MSCs grown to confluence on ITO substrates coated with a (PAH/PSS)<sub>9</sub> thin films at physiological pH = 7.4 (A). Stable attachment of PD-MSC sheet at pH=5 (B). Detachment of intact PD-MSC sheet after 2-3 minutes deposition of culture medium at pH=4**

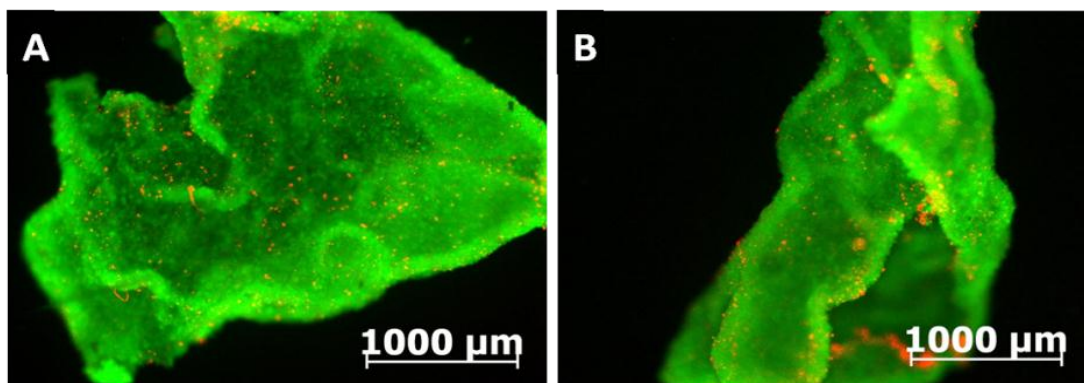
## 6.6. Characterization of recovered PD-MSC sheets

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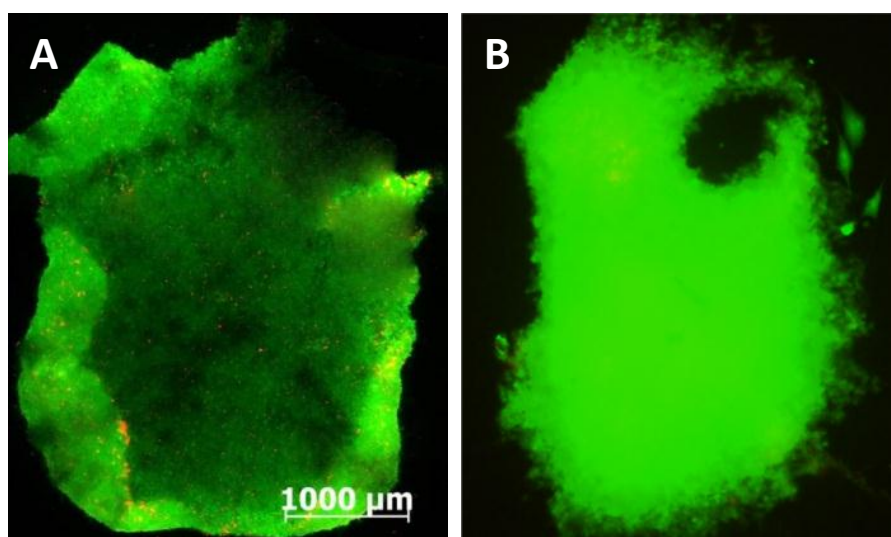
### Viability

The survival and functionality of PD-MSCs after release by electrochemistry and lowering of pH was tested immediately after the detachment by live-dead staining. As depicted, the live-dead staining performed within 2 hours after detachment of PD-MSC sheets, demonstrated that the cell survival was not significantly compromised **[Figure 50]**. Furthermore, the results confirmed that PD-MSC sheets, harvested upon electrochemical trigger or reduction of the bulk pH (data not shown), retained their potential for adhesion and growth on novel TCPS substrates **[Figure 51 A]**.

Normally cell sheets manually transferred after electrochemical detachment started to adhere to fresh TCPS substrate within approximately 48 hours. Within next 7 days the first signs of cell sheet growth appeared **[Figure 51 B]**.



**Figure 50: Viability of stem cell sheets after peeling.** Viability of the cell sheets peeled by electrochemical stimuli (A), and decrease of bulk pH (B) as demonstrated by Live-Dead staining with calcein (green) specific for live cells and ethidium homodimer [220] specific for dead cells.

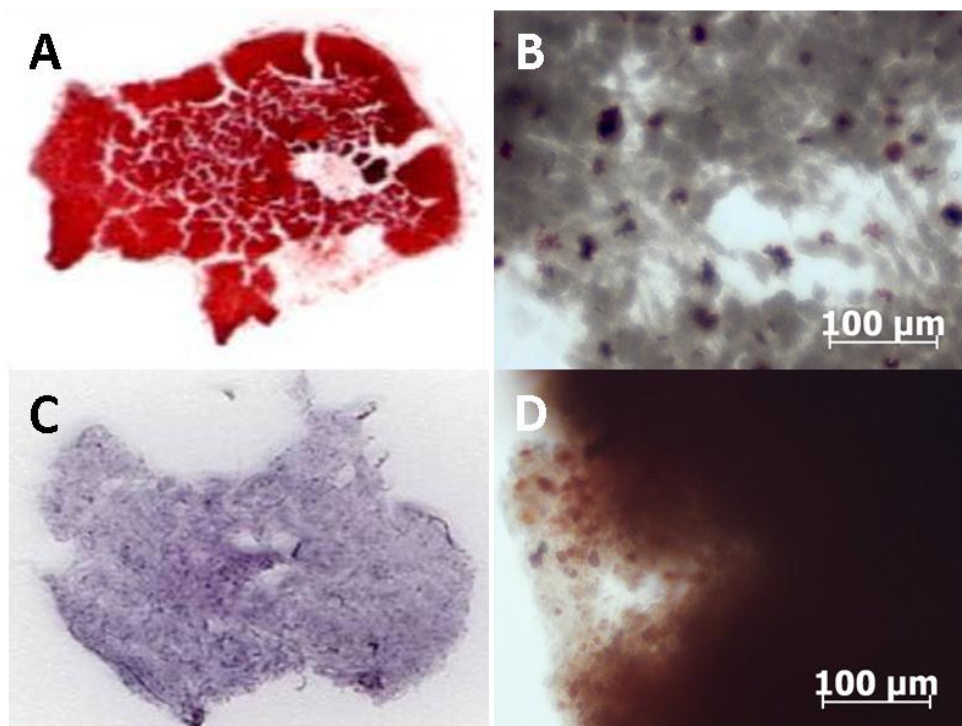


**Figure 51: Viability and adhesion capacity of transferred stem cell sheets.** Viability of electrochemically peeled PD-MSC sheet (A) and their attachment to the TCPS surface after 7 days of culture (B) as demonstrated by Live-Dead staining with calcein (green) specific for live cells and ethidium homodimer [220] specific for dead cells.

#### **Differentiation towards mesoderm induced by medium**

Differentiation of recovered PD-MSC sheets towards adipogenic and osteogenic lineages *in vitro* could be successfully shown by Oil Red O and Alizarin Red S staining, respectively [Figure 52]. The efficiency of this differentiation was comparable with the

results obtained after mesodermal differentiation of confluent cell layers grown on (PAH/PSS)<sub>9</sub> substrates deposited on ITO coated glass electrodes described above. Chondrogenic differentiation was not detachable.



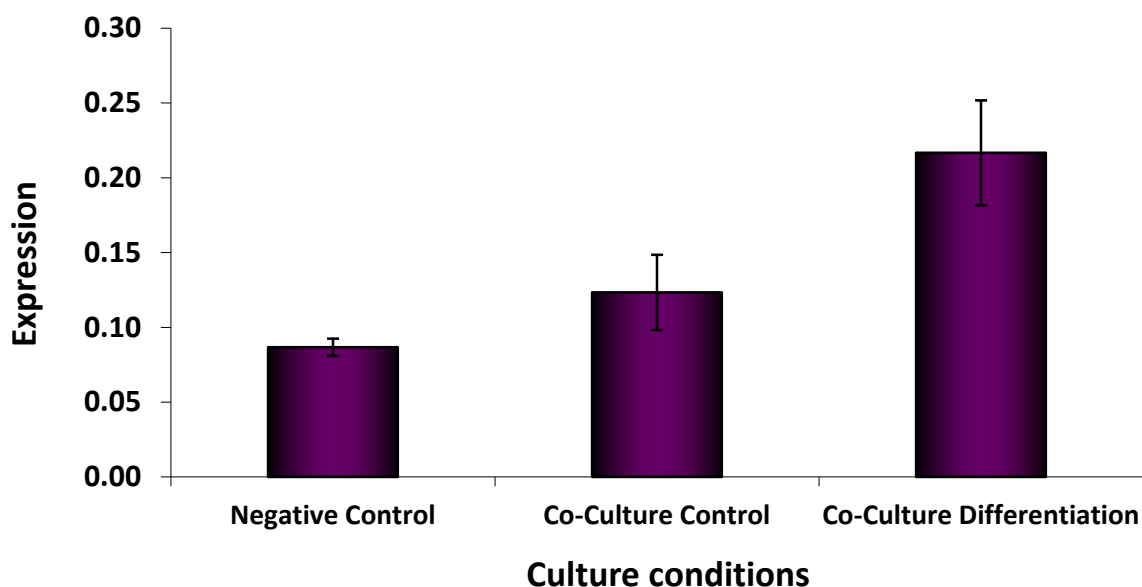
**Figure 52: Mesodermal plasticity of stem cell sheets after peeling as demonstrated by appropriated biochemical stainings. Osteogenic differentiation as demonstrated by Alizarin Red S-staining of calcification after electrochemically mediated peeling (A) and colorimetric staining of alkaline phosphatase after pH mediated peeling (C). Adipogenic differentiation as demonstrated by Oil Red O-staining of lipid droplets after electrochemically mediated peeling (B) and after pH mediated peeling (D).**

#### **Osteogenic differentiation of PD-MSC sheets by direct cell contact**

Finally, I demonstrated that recovered PD-MSC sheets retain potential to be differentiated towards osteogenic lineage by co-culture in direct cell-cell contact with human osteoblasts **[Figure 53]**. The ranges of collagen I expression by negative control, co-culture control and co-culture differentiation conditions were significantly different. The efficiency of this differentiation was comparable with the results obtained



after co-culture of PD-MSC sheets with human osteoblasts in the absence of cell-cell contact.



**Figure 53: Differentiation of recovered PD-MSC sheets towards osteogenesis by co-culture as determined by expression of human collagen type I using real-time PCR.**

### 6.7. Relevance (PAH/PSS) substrates for stem cell sheet engineering

In the present study, a new platform for the growth and subsequent harvesting of stem cell sheets was explored. Robust (PAH/PSS) thin films were used as substrate for PD-MSCs, and viable stem cell sheets were effectively released from the polyelectrolyte substrates through a local, electrochemically-induced pH lowering or a global pH decrease of the bulk solution. (PAH/PSS) multilayers promoted the formation of confluent PD-MSC monolayers while preserving the viability and differentiation potency of the adult stem cells. The stem cell sheets harvested upon electrochemical trigger or reduction of the bulk pH were able to adhere onto new TCPS surfaces, were viable, and could be successfully differentiated towards adipogenic and osteogenic lineages. In a previous work, native multilayers of poly-(L-lysine) and hyaluronic acid (PLL/HA) were shown to be poorly adhesive for PD-MSCs, despite efficient functionalization by adsorbed fibronectin, while highly cross-linked, stiffer (PLL/HA) multilayers were

proved suitable substrates for MSC adhesion and proliferation. [221] PLL and HA are weak polyelectrolytes which form relatively soft and highly hydrated coatings. Contrastingly, (PAH/PSS) assemblies present a dense, stratified structure resulting in much stiffer films, and a variety of other cell types, including stem cells, have been successfully cultured on (PAH/PSS) substrates [207, 222-232]. In this work, (PAH/PSS)<sub>9</sub> films allowed for the adhesion and growth to confluence of PD-MSCs without affecting

the stem cell's viability, phenotype and multipotency, further substantiating the promising properties of (PAH/PSS) coatings for biomedical applications. Placental MSC sheets grown on (PAH/PSS)<sub>9</sub> films completely detached within 10 to 20 minutes when subjected to a current density of 30  $\mu\text{A}/\text{cm}^2$ . Parallel, EcAFM experiments showed that the same electrochemical stimuli applied for 30 minutes was not sufficient to induce the coating dissolution, in good agreement with a previous report which showed a delayed, slow dissolution detectable after only 120 minutes at similar electrochemical conditions [233]. According to previous studies, the electrochemically-induced degradation of polyelectrolyte multilayers relies on the neutralization of the electrostatic interactions within the assemblies, as a consequence of water electrolysis which results in a continuous production of protons at the electrode surface as it is depicted on the schema:



Due to the low pKa value of PSS sulfonate groups (pKa  $\sim$ 1), the destabilization of (PAH/PSS) assemblies requires a very high concentration of protons, hence its delayed, slow dissolution. The dissolution of polyelectrolyte assemblies has been previously shown to be dependent on the intensity of the electrochemical stimuli applied, with higher potentials/currents yielding faster dissolution. The stability of (PAH/PSS)<sub>9</sub> thin films was thus assessed under a higher current density. The integrity of the thin films was maintained for at least 30 minutes under the increased electrochemical stimulus. These results clearly demonstrate that the cell sheet detachment observed in this study did not rely on the dissolution of the cell's underlying substrate, in contrast to the former electrochemical approaches for cell sheet engineering.

While the applied electrochemical trigger was insufficient to induce the degradation of the (PAH/PSS) thin films, it has been previously evidenced that the pH lowering induced by the electrochemical reaction can extend above the film. Indeed, weak (PLL/HA) assemblies, more sensitive, deposited on the top of a (PAH/PSS) thin film were shown to dissolve readily upon electrochemical trigger [233]. It can thus be assumed that in this work the pH at the film/cells interface decreased upon electrochemical stimuli. The interface between the cells and the substrate consists of a rich and complex protein layer, and a change in pH may greatly affect the protein-protein or protein-substrate interactions, and the individual protein conformations. Such perturbations in the protein layer on which the cells are adhering may induce the detachment of the cells. To verify this hypothesis, the effect on cell adhesion of a decrease in the bulk pH was evaluated. An environmental pH of 4.0 was found to induce a rapid detachment of the cell sheets, while pH comprised between 7.4 and 5.0 had no effect on the cells attachment on (PAH/PSS) substrates.

The integrity of (PAH/PSS)<sub>9</sub> films immersed in a solution at pH 4.0 was confirmed by AFM measurements, excluding a substrate dissolution as a cause of cell detachment. These results confirmed that the cell sheet detachment was induced by a lowering of the pH, either local (electrochemically-induced) or global. The electrochemically-induced pH lowering led to a slower cell sheet detachment compared to a direct change of the cover medium pH, a delay most probably due to the time necessary for the production of a sufficient amount of protons to reach a pH of ~4 above the polyelectrolyte substrate. It is well known that local pH changes in a cell micro-environment can significantly affect final population density, reversal of contact inhibition of growth, or efficiency of cell fusion *in vitro* [234]. For this reason, cell surrounding pH must be kept within relatively narrow limits for the maintenance and stable growth of most cell cultures. As previously reported by Taylor et al., the elevation of the environmental pH can lead to acceleration of cell movements and cause cytoplasm contraction, while the lowering of pH can retard and stop cell activity, causing apparent gelation of the protoplasm [235]. Earlier Cecarrini and Eagle already demonstrated that the growth rate as well as the maximum population doubling of whether normal, cancer, or virus-transformed cell

types were markedly pH dependent. The optimal pH ranges varied from 6.9 for the rabbit lens cells, to 7.8 for human fibroblast [236].

The effect of electrochemically-induced pH lowering on the viability of cells grown directly on an electrode has been studied by Gabi et al. Results showed that current densities up to  $38 \mu\text{A}/\text{cm}^2$  did not significantly affect cell viability, while  $58 \mu\text{A}/\text{cm}^2$  induced transient pore formation in the cell membrane, and an electrochemical trigger  $\geq 76 \mu\text{A}/\text{cm}^2$  induced cell death. They also showed that a  $\text{pH} \leq 2$  induced cell death [237].

## 6.8. Achievements of the sixth part

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In the third part of my thesis I evaluated potential usability of (PAH/PSS) polyelectrolyte multilayer substrates for engineering, differentiation, and controllable, electrochemically mediated detachment of the PD-MSC sheets. For this I assessed the effect of both local and global pH reduction on the viability and differentiation potential of the PD-MSC sheets. Live-Dead staining as well as differentiation assays of the harvested MSC sheets demonstrated that the pH decrease inducing the cell sheet detachment had no adverse effects on the cells. Beside their good properties for cell culture, the thin (PAH/PSS) films might also shield the cells from reactive products produced by the electrochemical reactions at the electrode surface. Indeed, beside reactive oxygen produced upon water electrolysis, the electrochemical stimuli may also induce the formation of amine reactive hypochloric acid [237]. These highly reactive moieties could have cytotoxic effects on cells attached directly on the electrode. However, these products cannot diffuse through a (PAH/PSS) film but will react and be neutralized at the electrode/polyelectrolyte interface.

# CONCLUSIONS

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Despite its remarkable progress, regenerative medicine is still faced with critical challenges such as correct tissue integration and survival as well as prevention of rejection and inflammations. Today's regenerative medicine strategies rely on the use of cells, biomaterials, growth factors and cell culture systems to form tissue replacements *ex vivo* or stimulate tissue regeneration *in situ*. New research efforts are currently directed towards the formation of well-tolerated, viable, and functionally-integrated tissue structures.

My project focuses on the formation of scaffold-free stem cell assemblies. In context of the project a highly-reproducible, straightforward method for the isolation of multipotent MSCs from human term placentas was developed and an approach based on conductive polyelectrolyte multilayer (PEM) coatings for the electrochemically-mediated harvest of placental mesenchymal stem cell sheets was established. These coatings turned out to be suitable substrates for such human end-differentiated cells as chondrocytes, osteoblasts, and smooth muscle cells. Moreover they permitted the formation of cell sheets using such human stem cell sources as placenta-derived MSCs, adipose tissue-derived MSCs, and late outgrowth endothelial progenitor cells.

Obtained results demonstrated that the mechanical behaviour of the PEM coating specifically influenced the efficiency of cell adhesion. Further, cell sheet peeling can be achieved through an electrochemically-induced local pH decrease.

As AFM results do not show any sign of the coating disintegration, this suggests that the recovered cell monolayers are free of polymer, which is desirable for potential clinical applications. Furthermore, the integrity of the PEM substrate after cell sheet detachment is promising when envisioning multiple uses of the same substrate.

The cell sheets detached as a result of electrochemical stimulus, even in absence of surface dissolution. This effect might be due to electrochemically-induced reduction of the local pH. Since cell sheet release could be reproduced by low environmental pH, the electrochemically-induced release could be attributed to a local drop in pH.

I propose that, reasonably-priced PEM-based systems might be used for the generation of cell layers with site specifically released or modified areas or could be explored to form cell sheets that consist of different cellular components that can be patterned in

2D. Specific deposition of endothelial cells might allow for the construction of vascular trees spanning the 3D constructs, which were formed by multiple cell sheets. Such biomaterial-free tissue replacements would address at least two of the tissue engineering challenges (neovascularization and correct tissue integration) therefore improving the integration and reducing the adverse immunological properties of such tissue-like structures.

Further studies are required to evaluate the properties of PD-MSCs regarding their full differentiation capacity *in vitro* and *in vivo*. Aside from evaluating their utility in future cell replacement therapies and tissue engineering, it is important to assess PD-MSCs potential for exploitation in transplantation medicine. It would be most favourable if PD-MSCs could exert immunosuppressive effects and/or secrete proangiogenic and antiapoptotic cytokines for the promotion of tissue healing. Furthermore, it will be important to evaluate the potential of (PAH/PSS) coatings as a carrier-substrate for the eventual triggering of growth factors, hormones, or other agents that stimulate stem cell differentiation.





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## 8.2. ABBREVIATIONS

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### Cell culture techniques

MSC – Mesenchymal Stem Cells

PD-MSC – Placenta-derived Mesenchymal Stem Cells

AT-MSC – Adipose Tissue-derived Mesenchymal Stem Cells

HUVEC – Human Umbilical Vein Endothelial Cells

NHDF – Normal Human Dermal Fibroblasts

OEC – Late Outgrowth Endothelial Cells

TCPS – Tissue Culture Polystyrene

HCH – Human Chondrocytes

HOB – Human Osteoblasts

HF – Human Fibroblasts

C2C12 – Mouse Muscle Cell Line (*Mus Musculus*)

FN – Human Recombinant Fibronectin

ALP – Alkaline Phosphatase

BCIP/NBT – (5-Bromo-4chloro-3-indolyl phosphate/Nitro Blue Tetrazolium)

### Polymers

PEM – Polyelectrolyte Multilayer

PLL – Poly-L-Lysine Hydrobromide

HA – Hyaluronic Acid Sodium Salt

PAH – Poly(allylalanine)Hydrochloride

PSS – Polystyrene Sulfonate

### Crosslinkers and buffers

HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HEPES-2 - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with 1.8 Mm NaCl

EDC - 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimid

NHS - N-hydroxysulfosuccinimide

**Analytical techniques**

FACS – Fluorescent Activated Cell Sorting

FITC / TRITC – Fluorescein Isothiocyanate / Tetramethylrhodamine Isothiocyanate

PE – Phycoerythrin

APC – Allophycocyanin

PCR – Polymerase Chain Reaction

AFM – Atomic Force Microscopy

OWLS – Optical Wave Length Spectrometry

### 8.3. CURRICULUM VITAE

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Since 2007	Swiss Stem Cell Network

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## Articles

- 2009                      **Semenov O.V.**, Malek A., Bittermann A-G., Voros J., and Zisch A.H. Polyelectrolyte Multilayer Substrates for Adhesion, Expansion, and Differentiation of Human Mesenchymal Stem Cells; Tissue Engineering Part A 2009 Oct; 15(10): 2977 – 90.
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- \*Both authors contributed equally to this work*

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2007

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Harmsen M.C., Zisch, A.H., Critical parameters for serum-  
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05.2010	Annual Meeting of Swiss Society of Biomaterials (SSB), Dübendorf, Switzerland
06.2010	Tissue Engineering and Regenerative Medicine International Society (TERMIS) European Union, Galway, Ireland
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